

**PROLIFERATION AND DIFFERENTIATION
OF GRANULOPOIETIC CELLS
IN CHRONIC MYELOID LEUKEMIA**

Bo Nilsson



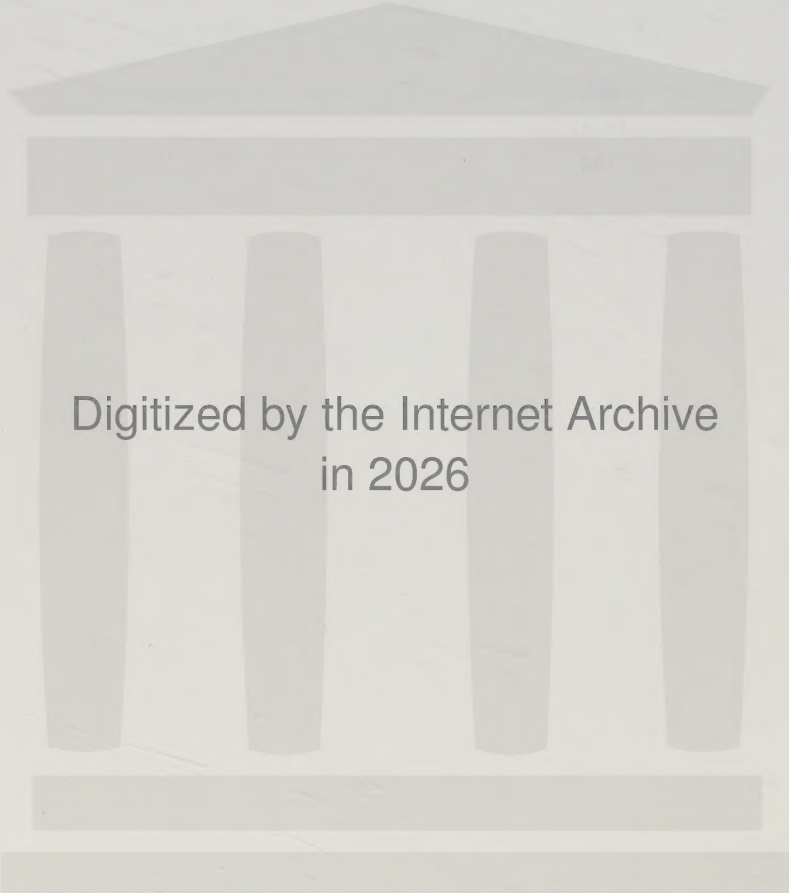
Lund 1985

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This thesis is based on the following papers, which will be referred to by their roman numerals.

- I. Nilsson B, Olofsson E, Olofsson T, Olsson I.
Proliferation and differentiation in diffusion chambers of marrow, blood and spleen cells from patients with chronic myeloid leukemia during chronic phase and blastic transformation.
Scand J Haematol 1980; 24:381-92.
- II. Nilsson B, Olofsson T.
Proliferation and differentiation of normal and chronic myeloid leukemia (CML) marrow cells in suspension cultures.
Scand J Haematol 1984; 32:321-40.
- III. Nilsson B, Olofsson T, Olsson I.
Myeloid differentiation in liquid cultures of cells from patients with chronic myeloid leukemia: effects of retinoic acid and indomethacin.
Exp Hematol 1984; 12:91-99.
- IV. Nilsson B, Olofsson T.
Density distribution of chronic myeloid leukemia and normal colony-forming cells in diffusion chambers (CFU-D) and agar (CFU-GM).
Scand J Haematol, accepted for publication.
- V. Nilsson B, Olofsson T.
Defective recloning capacity of granulocyte-macrophage colony-forming cells in chronic myeloid leukemia.
Submitted for publication.
- VI. Nilsson B.
Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukemia.
Br J Haematol 1984; 57:365-71.

ABBREVIATIONS

CML	chronic myeloid leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
CFU-S	colony-forming unit-spleen
CFU-GEMM	" -granulocyte, erythrocyte, megakaryocyte, monocyte/macrophage
CFU-D	" -diffusion chamber
CFU-GM	" -granulocyte, monocyte/macrophage
CFU-MK	" -megakaryocyte
BFU-E	burst-forming unit-erythroid
CSA	colony stimulating activity
CSF	colony stimulating factor
PMN	polymorphonuclear neutrophil
WBC	white blood cell
Ph ¹	Philadelphia (chromosome)
DC	diffusion chamber
RA	retinoic acid
HP-CM	human placenta-conditioned medium
PGE	prostaglandin E
FBS	fetal bovine serum
S-phase	DNA-synthesis phase of the cell cycle

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INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal disease which arises in a pluripotent stem cell (1) and has a specific chromosomal abnormality -the Ph¹-chromosome (2). The Ph¹-chromosome occurs as a consequence of a translocation between chromosomes 9 and 22 (3), and serves as a ground for the classification in typical, i.e. Ph¹-positive, and atypical or Ph¹-negative CML. The chronic phase of the disease is characterized by a greatly expanded granulopoiesis. During this phase, hemopoietic cell maturation seems to be relatively normal. However, intraclonal heterogeneity and malignant evolution often leads to a metamorphosis into acute phase CML characterized by a cell differentiation arrest similar to that in acute leukemia.

The chronic phase of the disease is commonly treated with busulphan or hydroxyurea, which gives symptomatic relief but not significant prolongation of life of CML patients. Results of chemotherapy of the acute phase have been largely disappointing. The use of bone marrow transplantation has provided new hope for a small group of young CML patients to whom a successful transplantation from an HLA-identical donor may result in a definitive cure.

The main purpose of this thesis has been to try to define abnormalities of proliferation and differentiation in CML.

PREVIOUS INVESTIGATIONS

The stem cell concept

a) General remarks

The blood cell compartments are preserved by stem cells, which have a capacity for self-renewal necessary for the maintenance of a continuous provision of marrow progenitors. Stem cells can be committed to differentiation into either of the blood cell lineages (4). Stem cells cannot be identified by light microscopy. Instead, so-called clonogenic assays are used for their identification based on colony formation upon culture in a semisolid milieu in the presence of specific growth factors (see below). (Fig 1).

Regulation of proliferation and differentiation of stem cells can be described as a stochastic process (5), independent of exogenous signals. If the probability for differentiation is P_1 the probability for self-renewal will be $1-P_1$. Obviously the value of P_1 has to be lower than 0.5 if the stem cell pool is to be preserved; otherwise the individual will run out of stem cells and lose the capacity for blood cell production. Environmental factors such as specific growth factors and influence from the microenvironment (6) may, however, change the probabilities for self-renewal and commitment to differentiation and thus participate in the regulation of hemopoiesis.

Stem cells seem to have an age structure. P_1 increases with increasing maturity of the stem cell (7). As the organism ages, more immature stem cells seem to maintain hemopoiesis compared to younger organisms (8). Serial transfer of stem cells to repopulate hemopoiesis can only be made a limited number of times in lethally irradiated mice (9). Thus even if stem cells have a very high capacity for proliferation they are not immortal.

b) Colony-forming unit-spleen (CFU-S)

The CFU-S denotes the multipotent, most primitive stem cell, that is demonstrated by the injection of murine bone marrow intravenously into lethally irradiated syngeneic mice with the subsequent formation of colonies of mixed hemopoietic cells in the spleen (10). Large colonies become macroscopically visible on the surface of the spleen within 7-10 days after infusion of marrow cells, and may be composed of neutrophilic, eosinophilic, erythroid, megakaryocytic and undifferentiated cells (11). Results from studies of chromosome markers have demonstrated that a colony is derived from a single CFU-S (12-14).

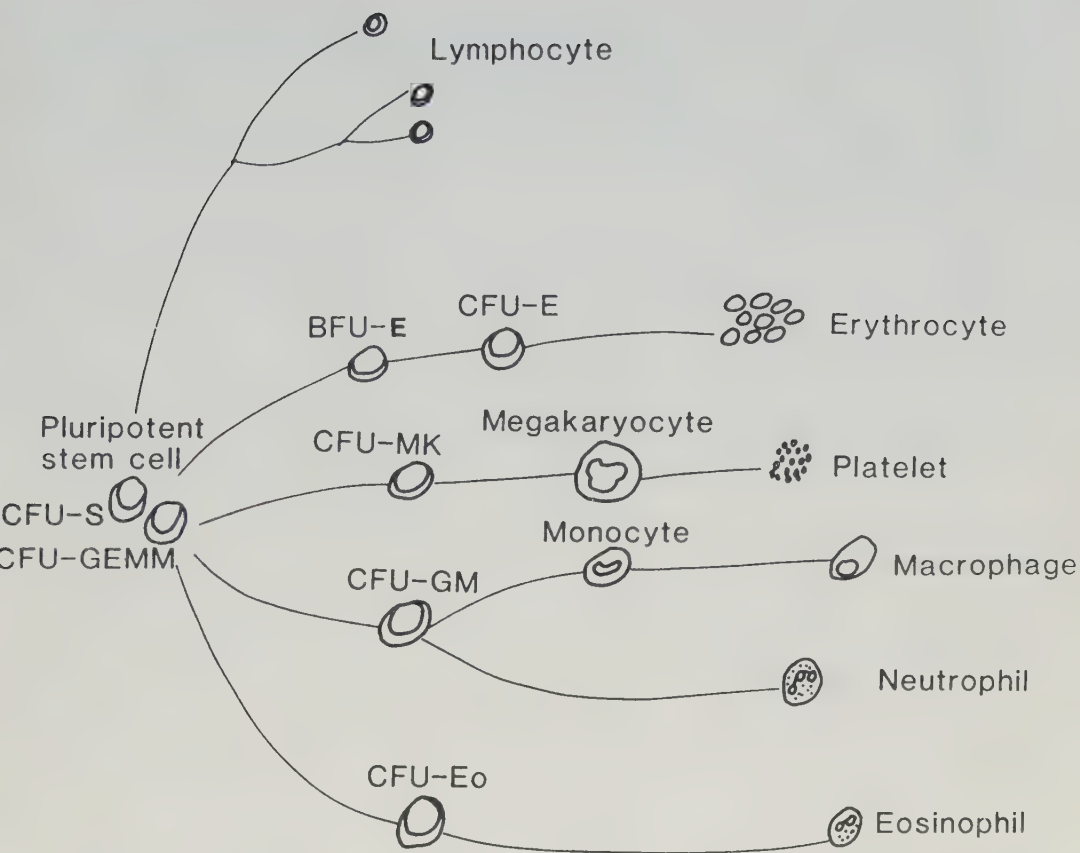


Fig. 1

Blood cells are produced from stem cells, which are defined as colony forming units (CFU) in vitro. For details, see text.

The mixed colonies of the spleen contain mostly erythroid cells, whereas mixed colonies of the marrow contain predominantly granulocytic cells. Thus different colony types are non-randomly distributed between the bone marrow and the spleen, suggestive of an environmental influence, from the "hemopoietic inductive microenvironment" (11, 15).

At steady state only about 10% of the CFU-S's are synthesizing DNA, but during regeneration the proliferation is augmented such that 30 to 40 per cent of the CFU-S are in DNA-synthesis (S-phase) (16, 17).

The CFU-S has a high capacity for self-renewal as would be expected for a multipotent stem cell (18). Thus a single spleen colony may contain from a few to several hundred daughter CFU-S, with a distribution in individual colonies in accordance with the stochastic model of hemopoiesis (5). According to this model, the choice of the stem cell between self-renewal and differentiation is determined by chance only.

c) Colony-forming unit-granulocyte, erythrocyte, megakaryocyte, monocyte/macrophage (CFU-GEMM)

Human pluripotent hemopoietic progenitors can be identified by their ability to give rise in methyl cellulose culture to mixed colonies that contain cells from several lineages of hemopoiesis (21-25).

Less than 10% of CFU-GEMM from normal marrow are in DNA-synthesis (22, 25). The proliferation of CFU-GEMM can be almost completely inhibited by prior incubation of cells with cytotoxic antibodies with DR specificity, indicating that the pluripotent stem cell carries HLA surface determinants (26, 27). The CFU-GEMM is a non-adherent cell with a density lower than 1.077 g/ml, and is a relatively small cell judging from sedimentation velocity in albumin gradients.

The CFU-GEMM can be recovered in the non-T lymphocyte fraction of marrow cells (28).

The proliferation of the CFU-GEMM is increased during marrow regeneration and after exposure to isoproterenol, thus indicating a similarity to murine CFU-S (28).

The pluripotency of CFU-GEMM is demonstrated in experiments where replating produces secondary hemopoietic colonies containing both CFU-GEMM, BFU-E, CFU-E and CFU-GM (28-30).

d) Colony-forming unit-diffusion chamber (CFU-D)

The formation of mature granulocytes and macrophages during culture of mononuclear blood or bone marrow cells in diffusion chambers implanted intraperitoneally in mice was described in 1970 (31, 32). This technique has been improved to allow studies of colony formation in diffusion chambers containing plasma clots or agar (33, 34) where the formation of granulocyte or macrophage colonies in these chambers defines a hemopoietic stem cell designated CFU-D. Differences between subpopulations of CFU-D have been reported with regard to cell cycle activity, size, resistance to osmotic lysis and the morphology of the granulocyte colonies to which these cells give rise, suggesting that the CFU-D population is heterogenous (35, 36). Most CFU-D do not seem to be in active cell cycle since the fraction in S-phase is lower than 10% (37). DR antigens are regularly expressed on CFU-D (38, 39).

The number of CFU-D in normal human bone marrow has been estimated at 7-80 per 10^5 low density cells (37, 40). The colony formation in diffusion chamber is increased by pretreatment of the host mice with irradiation (41) or cyclophosphamide (42), indicating loss of inhibitory agents or enhancement of growth factors. Treatment of the host mice with endotoxin increases the size of the colonies (43).

The physical characteristics of CFU-D differ from those of CFU-GM (37, 44), and a parent-progeny relationship has been proposed between CFU-D and CFU-GM, where CFU-D should be the precursor cell for CFU-GM (45).

Thus CFU-D may represent a hemopoietic stem cell committed to development along the granulocytic and monocytic lineages but more immature than the CFU-GM.

e) Colony-forming unit-granulocyte-monocyte/macrophage (CFU-GM)

Granulocyte production is maintained along a one-way pathway, where successive divisions of granulocyte precursors give rise to increasing numbers of more mature progenies. Granulopoiesis seems to be regulated by a number of factors, which act on both uncommitted and committed stem cells. Such factors can both stimulate and inhibit cell growth (see below).

The CFU-GM is defined as a clonogenic cell, that through proliferation forms colonies in semisolid media by the action of growth factors, termed colony stimulating factor (CSF) or colony stimulating activity (CSA) (46-48). The colonies consist of cells of the granulocytic and/or monocytic cell series.

Normal human bone marrow gives rise to 10-50 colonies per 10^5 nucleated marrow cells (with more than 40-50 cells per aggregate counted after 7-14 days of culture) (49-55), while the number of CFU-GM in the peripheral blood is lower than 10 per 10^5 nucleated cells (49, 54, 56-58).

The CFU-GM does not adhere to glass or plastic surfaces (59), has a boyant density of 1.064-1.068 g/ml (60, 61), a sedimentation velocity of 5-9 mm/h (37), no markers for T or B lymphocytes (62, 63), but expresses DR antigen (64, 65). In normal human marrow and peripheral blood 10-50% of CFU-GM are in S-phase (37, 60, 66). The population of CFU-GM is heterogenous with respect to cell size and growth characteristics in vitro (37, 67-69).

Regulation of granulopoiesis

A necessary requirement for in vitro growth of granulocyte and monocyte/macrophage colonies is the continuous presence of CSA (70). Human CSA is produced by a variety of cells, such as monocytes (48, 60, 71-74), lymphocytes (71, 75, 76), endothelial cells (77) and marrow adherent cells (78). Production of CSA by malignant cells has also been demonstrated (79-82). Human CSA consists of polypeptides with molecular weights of 25 000 - 40 000 with or without associated carbohydrate (71, 81, 83-87). There are forms of human CSA that appear to activate selectively different subpopulations of CFU-GM (68, 69, 88, 89).

CSA of serum rises markedly when there is an increased demand for neutrophil production. This is found e.g. in irradiation-induced neutropenia in mice (90), neutropenia induced by peritoneal lavages in dogs (91), or by injections of anti-neutrophil serum into rats (92). In cyclic neutropenia, serum and urine CSA are inversely related to the peripheral blood neutrophil counts and varies in parallel with the monocyte counts (93-95).

Endotoxin and lithium stimulate CSA release from monocytes and macrophages in vitro, increase serum and tissue CSA and stimulate granulocytopoiesis in vivo (96-106). Sensitized lymphocytes elaborate CSA in response to antigen challenge (107). Thus several observations suggest a role in vivo of CSA for the production of granulocytes, but such a role is not yet firmly established.

Normal human serum contains activity associated with lipoprotein that inhibits colony formation from bone marrow cells in vitro (108). However, the inhibitory effect in vitro is not specifically directed against CFU-GM and its role in vivo is questionable (109, 110).

Different cellular products inhibit the formation of granulocytes and monocytes in agar culture, diffusion chamber culture and liquid culture (109, 111-121). Granulocyte extracts containing low molecular weight "chalcones" inhibit DNA-synthesis of cells in growing granulopoietic colonies in agar (122, 123). Lactoferrin, which is found in the specific granules of mature neutrophils, inhibits, in its iron-saturated form, the release of CSA from monocytes and macrophages (111, 124-127). Therefore a role has been proposed for lactoferrin

as an inhibitor of granulopoiesis in vivo (128). It seems to inhibit the production or release of CSA from monocytes and macrophages but not from lymphocytes (125, 126).

On the other hand, transferrin, a metal-binding glycoprotein biochemically similar to, but antigenically distinct from lactoferrin, has little or no influence on CSA-production from monocytes and macrophages (125, 129-132). However, in contrast to lactoferrin it suppresses the CSA-production by activated T-lymphocytes (115).

Marrow and blood cells from patients with acute or chronic leukemia release a high molecular weight glycoprotein, that reduces the fraction of normal CFU-GM in S-phase (133, 134). This glycoprotein has a molecular weight of more than 500 000 daltons, with an active subunit of 150 000-170 000 daltons. Subsequent studies have shown that the inhibitor is produced by an unidentified normal non-T, non-B, non-adherent, non-phagocytic cell with a boyant density of 1.075-1.080 g/ml (135).

Mononuclear phagocytes produce acidic isoferritins, which inhibit colony formation from normal CFU-GM (114, 136). This suppression is restricted to the DNA-synthetic phase of the cell cycle, when Ia-like antigens are expressed (137).

Prostaglandins of the E type, that can be produced by monocytes and macrophages, inhibit monocyte/macrophage formaU-GM accomplished by suppression of the stem cell during the S-phase and by a decreased sensitivity of the CFU-GM to CSA (138-140).

Moreover, a subpopulation of lymphocytes, the natural killer (NK) cells, inhibit the proliferation of normal human CFU-GM in vitro (141).

Incubation of cells in liquid culture can be used for studies of cellular proliferation and differentiation and hematopoietic cell interactions (142-147). In the Marbrook chamber system, cells proliferate in a diffusion chamber in vitro, that is surrounded by (exchangeable) culture medium. Cell production was reported to be dependent on chamber diameter, exchange of the surrounding medium and removal of mature cells (143).

In the long term culture system of Dexter (146), hematopoietic cells can be maintained for months on a layer of adherent marrow cells with a continuous production of mature granulocytes and macrophages. Pluripotent as well as committed stem cells may be harvested from such cultures (146).

Terminal differentiation and maturation of granulocytes

The morphologically recognizable granulocytic cells of normal human bone marrow may be divided into two different compartments, one mitotic or proliferative and one of maturation and storage. Myeloblasts, promyelocytes and myelocytes constitute the mitotic compartment, in which the number of cell divisions has been estimated at between four and five (148). One myeloblast will thus give rise to 8 to 16 mature PMN's. The maturation-storage compartment of the bone marrow contains the nonreplicating metamyelocytes, band forms and segmented neutrophils. The myelocyte-to-blood transit time has been estimated at 2-14 days (148-151). The mature granulocyte reserve of the marrow contains many more cells than are circulating in the blood. Under certain stress conditions the maturation time may be shortened, and release into the blood may occur prematurely (148, 152, 153).

A model of progressive restriction of differentiation potential has been proposed according to which stem cells sequentially lose their ability to differentiate into other cell types and finally emerge as committed cells of a specific lineage (154). Studies of myeloid specific antigens on myeloid leukemia cells and experiments with differentiation induction of a leukemic promyelocytic cell line, HL-60, indicates, however, that bipotentiality might be preserved as far as to the promyelocyte level (155-159).

Chronic myeloid leukemia (CML)

a) Clinical traits

CML is a rare disorder, affecting slightly more than 1 per 100,000 population, males and females alike (160, 161). CML is exceptionally rare in childhood, and uncommon in the elderly; its peak incidence is in the middle age (161).

The onset is insidious with non-specific symptoms, such as fatigue, weight loss, sweating and dyspnea. The spleen is nearly always palpable at presentation and is often massive (161). Localized sternal tenderness may be present in 75% of the patients (162).

Examination of the peripheral blood film usually makes the diagnosis of CML obvious; granulocytes in all stages of development occur in profusion and appear to be normal in morphology. The mature elements are present in greatest numbers and the less mature in diminishing frequency. Myeloblasts and promyelocytes usually do not exceed ten per cent (163).

A moderate normochromic, normocytic anemia is commonly present at diagnosis. The anemia may be secondary to the excessive granulocytic proliferation in the bone marrow, although the correlation between the WBC counts and anemia is poor (161).

In Ph¹-positive CML the platelet counts are initially normal or elevated, and very high counts ($6-7 \times 10^{12}/l$) have been reported (164, 165). In spite of the thrombocytosis, thrombo-embolic complications are not regular features of the disease (165). The Ph¹ chromosome has been demonstrated in megakaryocytes (166), and studies of glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme types have provided evidence that the platelets belong to the neoplastic clone (1). Abnormalities of platelet function are frequent, but have usually shown no correlation with clinically apparent bleeding (167-171).

Typical CML is characterized by the presence of the Ph¹ chromosome in the hematopoietic cells (2). The Ph¹ chromosome occurs as the consequence of a translocation between chromosomes 9 and 22 (3). The human cellular homologue (c-abl) of the transforming sequence of Abelson murine leukemia virus (A-MuLV) was recently shown to be located on chromosome 9q34 and translocated to Ph¹ in all Ph¹-positive CML-patients investigated so far. This translocation is independent of the observation that the terminal part of chromosome 22 can be transferred to recipient chromosomes other than chromosome nr 9, which occurs in a minority of CML-patients (172, 173). The human homologue (c-sis) of the transforming sequence of Simian sarcoma virus (SSV) has been reported to be

translocated from chromosome 22 to 9q+ in patients with the classical type of Ph¹ translocation (174). However, the role for oncogene products in the development of CML remains unknown.

In about 80% of patients with CML, a terminal phase or metamorphosis supervenes (175, 176). The duration of the chronic phase is very variable, the median time being 30-40 months with a very wide range; some patients are diagnosed in the terminal phase, while some live for more than 20 years in the chronic phase (177-179). The terminal phase of CML is a very heterogeneous entity; only a part of the patients appear with a blastic crisis, other patients showing various forms of dyshemopoiesis (180-183). The terminal phase is rapidly fatal with a duration of usually 3 to 6 months (184).

Treatment of CML

The median survival for untreated patients was in 1924 reported to be 19 months (185). In 1902, splenic irradiation was introduced (186), and in 1953, busulphan treatment of CML was reported (187, 188). Busulphan is an alkylating agent that produces a high response rate in previously untreated patients, and has been reported to be superior to splenic irradiation (189), chlorambucil (190), 6-mercaptopurine (191) and cyclophosphamide (192). With busulphan, survival in CML is usually about 40 months (193). Similar survival times have been obtained with dibromomannitol (194-198) and hydroxyurea (199, 200). Early splenectomy does not prolong the chronic phase, nor does it improve duration or quality of survival after the onset of transformation (201, 202). Leucophoresis may be a valuable measure when rapid reduction of the leukocyte count is required, as in patients with leucostatic problems (203). It can also be useful for control of CML during pregnancy (204), but has probably no advantage over cytotoxic drugs in the treatment of the typical patient with CML (193).

With aggressive combination chemotherapy it has been possible to obtain a conversion to a normal karyotype in Ph¹-positive CML (204, 205). However, the "remissions" were relatively short-lived, and significant prolongation of survival could not be demonstrated with aggressive chemotherapy as compared to conventional treatment.

Results from treatment of CML in metamorphosis have been largely disappointing with limited responses of short duration; a possible exception is vincristine-prednisolone treatment of blastic crisis of lymphoid type, whereafter a second chronic phase may be established (193).

In some patients with CML, marrow cells have been collected for cryopreservation during the chronic phase, and in blastic transformation autologous marrow transplantation was performed. The second chronic phases were, however, of short duration and survival was not very much prolonged (193).

In recent years, allogeneic bone marrow transplantation has been performed in a number of patients with CML. Bone marrow transplantation appears to be a promising therapeutic modality for patients with CML in that it offers the possibility of cure (206). However, bone marrow transplantation is generally restricted to patients less than 40 years old, which leaves the majority of CML patients without this possibility.

In conclusion, many treatment modalities have been applied in CML but still today, there is no effective therapy to prolong survival for the majority of CML patients.

c) Progression of CML

Almost all patients with CML develop a metamorphosis of the disease after 30-40 months (207). Chromosome abnormalities additional to the Ph¹ chromosome appear at or prior to the metamorphosis in a nonrandom manner: the most common abnormalities are trisomy 8, followed by an extra Ph¹ chromosome and iso(17q) (208).

The metamorphosis is also predicted by an altered growth pattern of granulopoietic progenitor cells in agar. The cluster: colony ratio increases, and the total number of colonies decreases (209). During the chronic phase, the leukocytosis doubling time becomes shorter after each course of busulphan, from diagnosis to the time of blastic transformation (210, 211). Abnormally high platelet counts, decreased red cell counts or an abnormally short unmaintained effect of chemotherapy may also predict an early transformation (212-214). The terminal phase includes many varieties of abnormalities in blood and marrow cells, such as myeloproliferative acceleration, myelofibrosis, refractory anemia and thrombocytopenia or blastic transformation (183).

The blastic transformation may be described as the appearance in the peripheral blood and bone marrow of large numbers of blast cells, which usually resemble myeloblasts seen in AML (215), but in a number of patients they may have morphological or cytochemical features of lymphoblasts (215-217). In rare cases the blast cells may be predominantly myelomonocytic, erythroid (218) or megakaryocytic (219, 220).

Thus, blastic crisis of CML may be expressed in any one of the different hemopoietic lineages, emphasizing that CML originates in a pluripotent stem cell.

In vitro studies of hemopoiesis in chronic myeloid leukemia

CML arises in a multipotent stem cell, common to erythrocytes, megakaryocytes, granulocytes and monocytes/macrophages (1). The pathologic clone coexists, at least during the early part of the disease, with normal hemopoiesis (221),

which, however, does not seem to be regularly expressed, since usually only Ph¹-positive cells are demonstrated (208). After treatment with the achievement of normal WBC counts, Ph¹-negative cells can sometimes be found (222, 223). The lack of expression of normal hemopoiesis in CML may be due to the elaboration of leukemia-associated inhibitors, the production of which is stimulated by the leukemic cells, and that inhibit normal granulopoiesis (133, 134, 224).

In the chronic phase of CML, the pool of CFU-GEMM (human multipotential stem cells) is greatly expanded, and the proportion of actively proliferating CFU-GEMM is significantly increased (25, 28, 225).

The burst-forming unit-erythroid or BFU-E is greatly increased in the blood of CML patients, the number of BFU-E being proportional to the white cell counts (226-229). In patients with untreated polycythemia vera a fraction of circulating erythroid progenitor cells is capable of proliferation in vitro in the absence of erythropoietin (230, 231). An analogous population of erythropoietin-independent BFU-E has also been demonstrated in blood or marrow from some (228), but not from other CML-patients (229). The fraction of normal BFU-E in S-phase is about 35%, but in peripheral blood from patients with newly diagnosed CML, the fraction of BFU-E in S-phase is only about 15%, thus indicating a slower than normal proliferation of early erythroid progenitors in CML (25).

In chronic phase CML, the number of circulating megakaryocytes progenitors in blood, CFU-MK, is increased by a factor of up to 300 (232, 233). Moreover, abnormal ploidy levels have been observed for CML megakaryocytes in culture indicative of maturation abnormalities (233).

The numbers of CFU-GM in bone marrow or peripheral blood are increased to a higher degree than has been reported for other classes of stem cells (60, 234, 235), and the density of CFU-GM is decreased (60, 61). Results from in vitro studies have shown a low cluster to colony ratio, perhaps indicative of a high proliferative potential of CML granulopoietic precursor cells (209). The fraction of CFU-GM in DNA synthesis is decreased to half its normal value in CML. However, when leukocyte counts are reduced to normal by chemotherapy the S-phase fraction rises to the normal range (60).

The number of CFU-GM in CML is proportionally more increased than the WBC count. Presumably only a fraction of normal CFU-GM gives rise to colony formation in agar (236). It is possible, that CML-derived CFU-GM have a higher plating efficiency in agar than nonleukemic cells, but it is also possible that in CML more mature cells than CFU-GM (e.g. myeloblasts and promyelocytes) give rise to colonies in agar but that this is not the case in cultures with normal cells.

The total body pool of CFU-GM is obviously expanded in CML. This could be explained by an autonomous cell proliferation, increased growth stimulation or decreased growth inhibition.

CML leukocytes produce normal, increased (237) or decreased amounts of CSA (234). Furthermore, both increased (238) and decreased responsiveness to CSA has been reported in CML (239).

A granulocyte-derived low molecular weight substance, which inhibits CFU-GM in S-phase is equally effective on CML and normal cells (121).

Lactoferrin-mediated inhibition of granulopoiesis is reported to be defective in CML, which has been suggested to explain, at least in part, the granulopoietic expansion in CML (240-242).

An increased production of a "leukemia-associated inhibitor", LAI, (133, 134) is reported in CML. In contrast to normal, CFU-GM from patients with CML are resistant to the action of LAI (133, 135), which may lead to a proliferative advantage for leukemic cells as compared to normal cells.

Acidic isoferritins from monocytes inhibit colony formation from normal CFU-GM during the S-phase. Cells from patients with acute or chronic myeloid leukemia are not responsive to this inhibition, which is linked to the expression on the cell surface of Ia-like antigens. Such antigens are absent or diminished on cycling CFU-GM from patients with leukemia, which may explain the lack of responsiveness of leukemic cells to acidic isoferritins (114, 136, 137).

Prostaglandin E inhibits the proliferation of CFU-GM; however, CML-derived granulopoietic progenitors have a reduced sensitivity to prostaglandin-mediated inhibition. (138-140, 243-245).

T-lymphocytes from patients with immune severe aplastic anemia or pokeweed mitogen-primed normal T-cells inhibit normal but not CML CFU-GM (246).

Thus according to in vitro findings the proliferative advantage of the leukemic cells in CML may result from a decreased responsiveness to several factors which inhibit proliferation of normal granulopoietic progenitors. Gavosto demonstrated that the ^3H -thymidine labelling indices of CML myeloblasts was negatively correlated to the bone marrow myeloblast frequencies (247). The myeloblast labelling indices are also negatively correlated to the peripheral leukocyte count (248). These results indicate that 1) the granulopoietic hyperplasia is due to the increased number of myeloblasts and not to an increased myeloblastic proliferation rate, and that 2) the leukemic myeloblasts are subject to the action of growth regulators, although not effectively enough to control granulocyte production.

In contrast, labelling indices of myelocytes from CML patients do not differ significantly from normal ones, and show no correlation with the peripheral leukocyte counts (248, 249).

The regulation of granulopoiesis is thus deranged in several ways in CML; a common feature of the defective mechanisms of growth regulation is that they operate on an earlier level than the myeloblast.

Induction of differentiation of leukemic cells

A basic feature of the cells in acute leukemia or the blastic phase of CML is an apparent maturation arrest. During the chronic phase of CML, the cell differentiation and maturation seems to be rather normal, although functional defects of the cells can be detected (250-252). Several murine and human leukemic cell lines, which grow continuously in vitro have been used for exploration of differentiation in leukemia (253). The HL-60 line was established from a patient with acute promyelocytic leukemia (APL) (159). It consists of promyelocytes with a low spontaneous differentiation in cultures, but which can be induced to mature to granulocyte-like or monocyte-like cells by use of a wide variety of agents (253). Retinoic acid at a concentration of 1-10 μ M induces granulocytic differentiation in HL-60 (254) by an unknown mechanism. Using primary suspension cultures of fresh AML cells it was shown that cells from patients with APL responded with maturation to the addition of RA (255).

THE PRESENT INVESTIGATION

Aims of the thesis

In recent years, a number of techniques have become available for the study of hemopoietic cell proliferation and differentiation. These techniques have been used to a limited extent to elucidate basic pathophysiologic mechanisms in CML. An increased knowledge of the proliferation and differentiation of early CML hemopoietic cells is clearly of importance. Therefore, the following questions have been asked:

1. Do intrinsic abnormalities occur in the proliferation and differentiation of CML cells that can be discovered in diffusion chamber culture in vivo or in suspension culture in vitro?
2. Is it possible to overcome the maturation arrest of the blast cells in CML of blastic crisis during in vivo or in vitro culture?
3. Is it possible to modulate the differentiation pattern of CML cells in vitro by use of agents such as retinoic acid or indomethacin? Are the effects of such agents different in CML as compared to normal cells?
4. CFU-GM are greatly increased in number in the peripheral blood of CML patients, correlating to the WBC. Are the CFU-D increased in a similar manner? The density of CFU-GM is abnormal in CML. Is the density of CFU-D as well abnormal in CML?
5. Can the granulocytic hyperplasia in CML be explained by an abnormal self-renewal capacity of the granulopoietic stem cells?
6. A case report on the in vivo effect of RA in acute promyelocytic leukemia is included in this thesis. The following question was asked: Is it possible to induce differentiation in vivo of a population of maturation-arrested promyelocytes?

METHODS AND RESULTS

Paper IProliferation and differentiation in diffusion chambers of marrow, blood and spleen cells from patients with chronic myeloid leukemia during chronic phase and blastic transformation

In order to answer the question whether intrinsic abnormalities in the proliferation and differentiation of CML cells can be detected under culture conditions, such cells were studied during diffusion chamber (DC) culture.

The cells from CML patients and normal controls were separated on iso-osmolar Ficoll-Isopaque in order to obtain cell suspensions highly enriched for myeloblasts and promyelocytes. Of critical importance for the comparison between the cells from different sources is that the composition of the cell suspensions is comparable. Both normal and CML cells used were indeed highly enriched for myeloblasts and promyelocytes, which made up 30-50% of the cells used, but 20-50% lymphocytes were present in the normal cell suspensions. It was assumed, however, that these lymphocytes did not exert any major influence on the growth or differentiation pattern of the granulopoietic precursors.

The diffusion chambers were made up of two cell-impermeable filters that were glued to each side of a plastic ring, making up a volume of 140 μ l. 100 μ l of a suspension with $2.5-4.0 \times 10^6$ cells per ml was injected into the chamber, which was sealed and implanted intraperitoneally into cyclophosphamide-pretreated mice. Cells were harvested two to three times a week during three to four weeks. The cells were counted and differential counts were made.

In chronic phase CML, there was a higher granulocyte production in DC than in the controls from healthy individuals. Normal immature marrow cells produced very few mature granulocytes; macrophage formation predominated. Spleen cells from patients with CML in chronic phase produced very few granulocytes compared to simultaneously cultured cells from blood or marrow.

Thus it was possible to demonstrate differences in myeloid cell growth and differentiation between CML and normal cells during DC growth, indicating intrinsic abnormalities of CML cells.

Cells from patients with CML in blastic crisis did not proliferate as much as those from chronic phase CML during DC culture; a substantial number of the myeloblasts disappeared during the first five days of culture judging from the appearance of degenerated and vacuolated cells. However, cell maturation did occur in these cultures with an early appearance of mature PMN's but not of macrophages. These maturation-arrested myeloblasts could thus be induced to differentiate during DC culture, possibly under the influence of differentiation-inducing stimuli from the host mice.

Paper II

Proliferation and differentiation of normal and chronic myeloid leukemia (CML) marrow cells in suspension cultures

Suspension cultures were used to further elucidate abnormalities in proliferation and differentiation of CML cells. The cells were separated in Percoll for enrichment of immature myeloid precursor cells lighter than 1.065 g/ml. The major difference in composition between normal and leukemic cell suspensions obtained by this separation procedure was the lymphocyte content, which was on the average four times higher in normals (30% vs 7.5% for CML cells).

However, it was shown that at least T lymphocytes did not have any influence on the growth or differentiation pattern of normal cells (fig. 2). Thus the difference in proliferation and differentiation between normal and CML cells that was observed during culture was probably due to intrinsic abnormalities of the CML progenitor cells and not to a difference in T lymphocyte content.

The isolated low density cells were incubated in suspension cultures at different initial cell concentrations and cells were harvested two to three times a week for 12-16 days. At a low initial cell concentration (i.e. 10^5 cells per ml), human placenta-conditioned medium (HP-CM) markedly stimulated granulocyte production from both normal and CML cells.

At a high initial cell concentration (i.e. 5×10^5 cells per ml) the addition of HP-CM had but a minimal effect on granulocyte formation. In the absence of HP-CM, a moderate number of granulocytes developed in the CML cultures, but not in cultures of normal cells during incubation for 12-14 days. Macrophage production was stimulated to a moderate degree by HP-CM, both for CML and normal cells irrespective of the initial cell concentration.

The content of CFU-GM in the suspension cultures was assessed using agar culture with counting of the colonies (more than 40 cells) after 7 and 14 days. In the suspension cultures with normal cells, the number of CFU-GM increased initially with a peak after two days and then decreased. In clear contrast, CFU-GM of CML decreased steadily with time of incubation.

In order to identify the factor(s) in the HP-CM that was responsible for the stimulation of granulopoiesis in the suspension cultures with a low initial cell concentration, HP-CM was chromatographed on DEAE-Sephadex and Sephadex G-75. The fractions thus obtained were incubated with low density cells in suspension cultures at a low initial cell concentration. The maximum production of mature neutrophils and macrophages occurred in the fractions where the

Differentiation in suspension culture

CELLS

$\times 10^{-5}/\text{ml}$

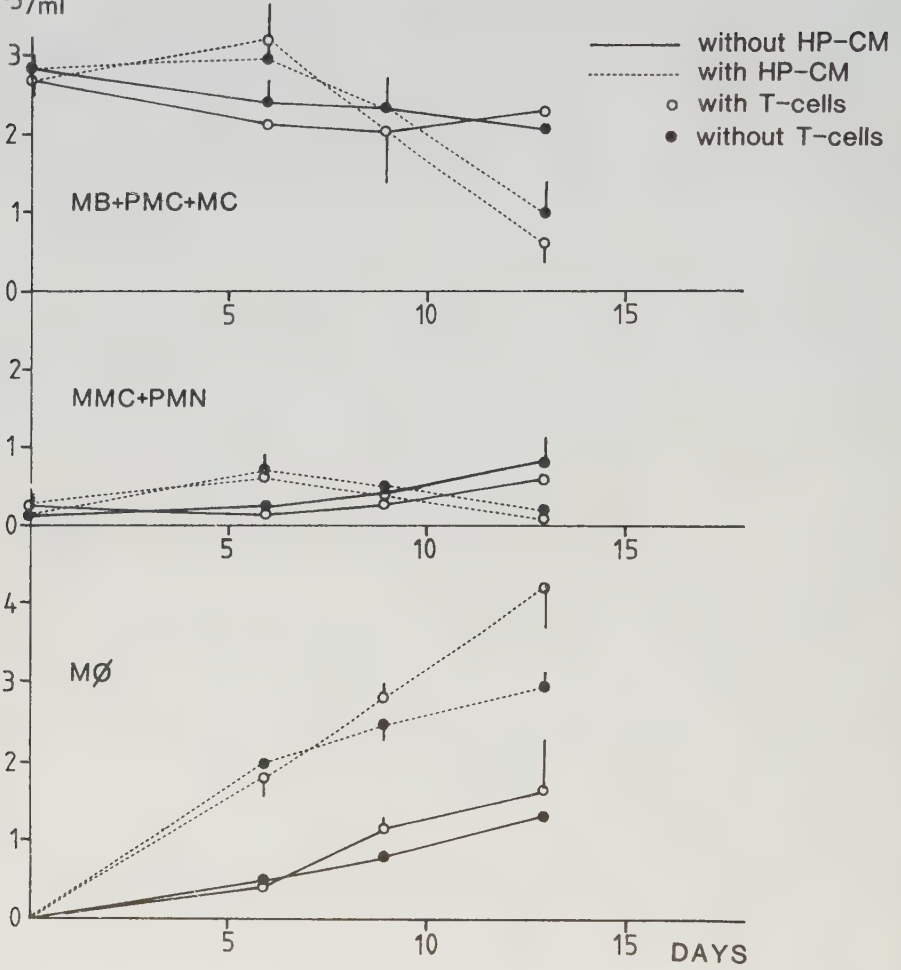


Figure 2. Removal of T lymphocytes did not change the pattern of differentiation of normal marrow cells in suspension culture.

greatest colony-stimulating activity was found, with similar results for normal and CML cells. Thus it seems probable, that colony-stimulating activity was responsible for the stimulation of granulopoiesis in the suspension cultures. In order to assess the role for intermediate and late granulopoietic precursor cells in the production of mature neutrophils, normal and CML cells with a density of 1.065-1.077 g/ml were incubated in suspension cultures. Such cultures were almost entirely devoid of colony forming cells; intermediate and late granulopoietic cells predominated. Moderate numbers of granulocytes were formed from both normal and CML cells; results were similar for high and low initial cell concentrations, respectively. HP-CM augmented the cell production to a lesser extent than that observed with low density cells. Thus it is likely that the bulk of neutrophil production in the suspension cultures was accounted for by immature granulopoietic cells, which also seemed to be the target cells for the action of HP-CM.

Paper III

Myeloid differentiation in liquid cultures of cells from patients with chronic myeloid leukemia: effects of retinoic acid and indomethacin

RA has been shown to induce differentiation in human leukemic cell lines, e.g. the promyelocytic cell line HL-60 and the histiocytic monoblastoid cell line U-937 (253, 256), and also in cells from patients with acute promyelocytic leukemia in primary culture (255). Furthermore, it has been reported that RA enhances colony formation in agar of normal marrow cells (257).

Prostaglandin E (PGE) inhibits the differentiation of CFU-GM along the monocytic pathway in vitro, but in CML, CFU-GM is less sensitive to this inhibition than normal stem cells (243-245). Therefore, indomethacin, that inhibits prostaglandin synthesis, might increase macrophage production from immature myeloid cells in suspension culture.

Normal and CML marrow cells were separated in Percoll gradients for enrichment of immature myeloid precursors lighter than 1.065 g/ml. Such cells were incubated in McCoy's medium with 15% FBS with or without 10^{-6} or 10^{-8} M of all-trans-retinoic acid or 10^{-6} M of indomethacin at a cell concentration of about 6×10^5 per ml. On subsequent days, cells were harvested for counting and differential counting, and on day 0 and day 2, cells were also harvested for assay of CFU-GM colony formation in agar. Colony morphology was assessed after fixation and staining of the agar gels.

RA, but not indomethacin, increased the total cell number in the liquid cultures, due to an increase of granulocytic cells. With RA, the number of macrophages was decreased compared to the controls. Indomethacin slightly decreased the number of granulocytic cells in the liquid cultures, and increased the macrophage number.

The effects of RA and indomethacin were similar on normal and CML cells. The number of colonies in agar culture was unaffected by RA and indomethacin, but in the presence of 10^{-6} M RA the number of clusters was markedly increased. Colony morphology was unaffected by the addition of RA or indomethacin with the exception of eosinophil colonies, that were decreased by RA and increased by indomethacin. The results were similar for normal and CML cells. CML colonies and clusters decreased during the first two days of liquid culture, while normal colony-forming cells increased, as described above (paper II).

At low and intermediate concentration of HP-CM, RA seemed to inhibit colony formation from CML cells but this inhibition was overcome at optimal concentrations of HP-CM. It is possible, that RA decreased the sensitivity of CFU-GM of CML to colony-stimulating activity.

A previous report showing an enhancement of colony formation in agar of normal marrow cells by RA was performed using cells separated in Isopaque - Ficoll gradients (1.077 g/ml) (257). The results from that study were confirmed, using the same method of cell separation. The increase of colony formation from cells lighter than 1.077 g/ml by RA (which is not seen from cells lighter than 1.065 g/ml) may be due to the influence of accessory cells on the CFU-GM or to colony formation from cells that usually do not give rise to colonies in agar.

Paper IV

Density distribution of chronic myeloid leukemia and normal colony-forming cells in diffusion chambers (CFU-D) and agar (CFU-GM)

During diffusion chamber (DC) culture, differences in the pattern of differentiation of immature myeloid cells from CML patients and normal controls could be demonstrated (paper I). In order to further define differences between normal and CML cells the work was extended to studies of colony formation in DC.

The CFU-D is a myeloid stem cell, that produces granulocyte-macrophage colonies in fibrin clots in diffusion chamber, that are implanted intraperitoneally into bone marrow depressed mice (33, 34). Previous data have indicated non-identity between the CFU-GM and the CFU-D based on findings of different kinetic properties (45) and sedimentation velocity as well as differences in the fraction of cells in active DNA synthesis (37).

Marrow cells were separated in linear gradients with Percoll, that were made from mixtures of Percoll and Hank's BSS, and formed by use of a peristaltic pump. Marrow cells were layered on top of the gradient and centrifuged; fractions were collected from the bottom of the tubes using the peristaltic pump. Thus, fractions of cells with stepwise decreasing density were formed. These cell fractions were used for the subsequent DC culture.

10^5 cells from each fraction were injected into each DC in a 0.5% fibrinogen solution, and a clot was formed by use of added thrombin. The DCs were implanted intraperitoneally into cyclophosphamide-pretreated mice, reimplanted into new cyclophosphamide-pretreated mice and the cells were harvested after a total of 14 days. After fixation and staining of the fibrin clots, the cell colonies were counted.

Cells from each fraction were also cultured in agar for assessment of the number of CFU-GM in the different fractions.

The CFU-GM had a homogeneous density distribution with colony-forming cells from normal controls in peaks at 1.060-1.063 g/ml and from CML patients in peaks at 1.058-1.061 g/ml. Thus CFU-GM from CML patients were lighter than their normal counterparts, as has been reported previously (61). The colonies were almost exclusively of pure granulocyte type.

The CFU-D on the other hand, had a heterogenous density distribution, both in normal and CML cultures. The colonies were of at least three different types with macrophage colonies from cells with the highest density, neutrophil colonies in low density fractions, or a minority of colonies with unidentifiable mononuclear cells.

In two CML patients, two different populations of CFU-D were found, one with a low and one with a high density, distinctly separated from one another. The nature of the high-density CFU-D in CML was further analyzed using a cell fraction obtained at 1.068-1.075 g/ml. Such cells were separated with regard to Fc receptors and cultured in DC. Both Fc+ and Fc- CFU-D were found. Plating efficiency was higher for both Fc+ and Fc- CFU-D than for a mixture of these cells, suggesting that interactions between these cell populations can modify the growth of CFU-D.

The total number of CFU-D in peripheral blood of patients with CML and normal controls was also determined in order to see if it correlated to leukocyte counts. For this purpose blood cells were separated in Isopaque-Ficoll (1.077 g/ml) and cultured in DC for assessment of CFU-D. In two patients with CML, the pool size of CFU-D was greatly increased in the presence of markedly elevated WBCs, while in two other CML patients with near normal WBCs, the pool size of CFU-D was normal.

It is concluded that cells forming colonies in DC are heterogeneous with respect to density and surface receptors. Suggestively, it is only those CFU-D with a density below 1.065 g/ml that represent stem cells, whereas colony forming cells with a higher density probably represent precursor cells with a limited proliferative capacity.

Except for increased concentrations of CFU-D in peripheral blood and bone marrow, CFU-D in CML were not different from normals.

Paper V

Defective recloning capacity of granulocyte-macrophage colony forming cells in chronic myeloid leukemia

When immature normal and CML marrow cells were incubated in suspension cultures, normal CFU-GM increased initially to a peak on day two of the culture, whereas CFU-GM from CML patients consistently failed to produce such an increase (paper II). This observation suggested either that normal CFU-GM expressed self-renewal capacity but CFU-GM in CML did not, or that normal marrow suspension cultures contained pre-CFU-GM capable to produce new CFU-GM. We decided to test the capacity for self-renewal of CFU-GM of normal and CML marrows.

For this purpose, marrow cells from healthy volunteers and patients with chronic myeloid leukemia in chronic phase were separated in Isopaque-Ficoll gradients (1.077 g/ml) and the low density cells were cultured in methyl cellulose with HP-CM as a source of CSA. After 7 days of culture, colonies were counted, and all the cells of the dishes were harvested after the addition of fresh culture medium. The cells were suspended, pooled, washed and replated in methyl cellulose as for the primary incubations. The recloning capacity was defined by dividing the number of secondary colonies with the number of replated primary colonies.

The normal recloning capacity was 3.84 ± 1.02 (SD). Control experiments indicated that the secondary colonies arose mainly from cells within the primary colonies and not from single cells in the culture dishes. Tertiary colonies were very seldom formed. CML colonies were defective in their recloning capacity, with values in general below 1.5. Six patients were studied shortly after the diagnosis of CML and before therapy had been started, and they all had markedly decreased recloning capacity. The defective self-replication was not related to WBC, duration of the disease or mode of treatment.

Subpopulations of CFU-GM have been defined, that form colonies after different length of time in culture; CFU-GM day 14 is assumed to represent a less mature granulopoietic stem cell than CFU-GM day 7 (37). Some data suggest that the capacity for self-renewal decreases with increasing maturity of stem cells (7). It can be argued, that the defective recloning ability of CFU-GM from CML

patients is due to an enrichment in CML of relatively mature CFU-GM with a low recloning ability. The normal CFU-GM day 14: CFU-GM day 7 - ratio in CML does, however, not support that interpretation.

CFU-GM day 14 and CFU-GM day 7 were separated by use of velocity sedimentation, and the highest recloning ability was found in fractions where CFU-GM day 14 predominated.

Paper VI

Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a subgroup of acute myeloid leukemia, usually characterized by bone marrow dominance of hypergranular promyelocytes, often containing multiple Auer rods (258). The disease is typically associated with disseminated intravascular coagulation (259, 260) and the marrow cells carry a unique chromosome abnormality in at least 50% of the patients, t(15q+;17q-) (261).

Retinoic acid has been shown to induce differentiation of human leukemic promyelocytes in vitro, such as the promyelocytic cell line HL-60 (254), or APL cells in primary suspension culture (255).

A 30-year-old woman with APL entered a complete remission after six courses of induction chemotherapy. She relapsed, however, after 2 months. A partial remission was obtained by reinduction therapy. This state was maintained by regular courses of daunorubicin (later abandoned because of high cumulated dose), ara-C and thioguanine. After eight months of partial remission, the number of promyelocytes in the marrow increased to more than 50%, and evidently, there was a block in the maturation of the cells. The chemotherapy was not changed, and after an observation period of four weeks, 13-cis-retinoic acid, 1 mg/kg was given orally. The number of promyelocytes in the marrow decreased to normal levels after 12 weeks, and the peripheral blood counts became normal. The treatment with intermittent courses of ara-C and thioguanine and with continuous RA has been maintained, and the patient is still in a complete remission 24 months after the start of retinoid therapy.

It is possible although unlikely, that the disappearance of promyelocytes during the administration of RA was spontaneous and part of the natural course of the disease. In addition, an effect of the chemotherapy administered cannot be completely ruled out. The long duration of the maturation arrest of promyelocytes in spite of continuous pulse chemotherapy favours, however, a role of RA as responsible for the differentiation induction.

GENERAL DISCUSSION

In this thesis, abnormalities of the production of granulocytes in CML have been demonstrated by use of culture techniques for immature myeloid cells. The studies have mainly been applied to cells from patients in the chronic phase of Ph¹-positive CML but cells from patients with CML in blastic transformation have also been studied.

A major objective was to obtain fractions enriched for immature granulopoietic precursor cells for the studies of maturation in DC and suspension cultures, and to ensure that as few mature cells as possible were present from the start of the culture period. This objective was achieved by density gradient centrifugation. The normal and CML cells isolated with this technique were comparable in morphological composition with the exception that lymphocytes were fewer in the CML specimens, but control experiments after removal of the majority of lymphocytes suggested that lymphocytes did not significantly alter the results. Another difference in cell composition was the higher concentration of colony-forming cells in the CML specimens, which may have contributed to the increased cell production observed for CML cells in DC and suspension cultures.

The finding in CML of a decreased capacity for differentiation with low final neutrophil maturation of spleen cells in DC as compared to marrow cells indicates intrinsic differences between spleen and marrow in this disease. The composition and kinetic properties of spleen and marrow cells in CML often show differences (249, 262). It is possible, that cells with properties different from those of the majority of the marrow cells are selectively retained in the spleen where the environment may be suitable for their proliferation. Moreover, the abnormal clone preceding blastic crisis can sometimes be found in the spleen before it is demonstrated in the marrow (263).

The lower granulocyte production in DC culture from blood compared to marrow cells in CML may be due to a mixture of marrow and spleen derived granulocyte progenitors in the peripheral blood, since the results for blood cells seem to be intermediate between those obtained for marrow and spleen cells, respectively.

An important finding was that myeloblasts from patients with CML in blastic crisis (CML-BC) matured in DC culture, although the production of mature PMN's was lower than for similar cells obtained during chronic phase CML. Thus the block in cell differentiation in CML-BC is not necessarily irreversible. A shortened maturation time as observed in the DC culture experiments may explain defective cytoplasmic maturation with granular abnormalities as observed in

vivo of the mature-appearing PMN's of CML-BC (251). Differentiation of AML blasts can also occur in DC culture (264). The maturation inducing factor(s) in the host mice responsible for these effects on AML or CML-BC blast cells have not yet been identified.

Because suspension culture in vitro could be subject to modulation more easily than DC culture in mice, the former was applied for additional investigation of growth and differentiation of myeloid progenitor cells. An important observation was the cell concentration dependency for growth in suspension cultures. At initial cell concentrations below 10^5 cells per ml, cell production was similar for normals and CML, but at initial cell concentrations of $4-8 \times 10^5$ cells per ml the CML cells still had the capacity for production of mature granulocytes whereas normal cells produced mostly macrophages. This pattern resembles that observed for cells grown in DC. At the higher cell concentration, CML cells in suspension culture were less dependent on exogenous CSA for granulocyte production than normal cells. These findings suggest differences between normal and CML cells that may be of importance for the pathophysiology of CML; first, CML cells do not respond normally to cell concentration dependent inhibition of growth and maturation, and second, CML cells may be less dependent on growth stimulating factors at increasing cell concentrations. It is an interesting observation in this context that erythroid progenitors in CML also have lower requirements for exogenous erythropoietin than their normal counterparts (228).

We studied the effects of RA and the PGE synthesis inhibitor indomethacin because of previously shown effects of these agents on proliferation and differentiation of hemopoietic cells. PGE inhibits the differentiation of CFU-GM along the monocytic pathway in vitro, but in CML, CFU-GM has a decreased sensitivity to PGE (243-245).

RA induces terminal differentiation in the human promyelocytic leukemia cell line HL-60 (254) and the histiocytic monoblastoid cell line U-937 (256). It also enhances colony formation in agar of normal marrow cells (257), which was confirmed in the present study when marrow cells lighter than 1.077 g/ml were used for culture. In contrast, when marrow cells lighter than 1.065 g/ml (retaining all colony forming cells) were used, no effect of RA on colony formation was observed. Therefore, it is likely that the stimulation of colony formation by RA is mediated indirectly through non colony forming accessory cells, which have a density higher than 1.065 g/ml and are absent in marrow cell specimens lighter than 1.065 g/ml. It is also possible, however, that RA stimulated colony formation from otherwise non colony forming cells with a density of more than 1.065 g/ml.

However, cluster formation from normal and - even more prominently -from CML cells was stimulated by RA, which resulted in a net increase in cell clones. The stimulatory effect of RA seems to act on cluster forming cells already committed to neutrophil production, and the most likely candidate for the effect would therefore be a cell within the myeloblast -promyelocyte compartment. The RA mediated increase of the number of both immature and mature granulocytic cells in suspension culture, and the decrease in production of macrophages is consistent with the enhancement of neutrophil cluster formation seen in agar culture.

An altered commitment of CFU-GM in response to RA or indomethacin could not be demonstrated; the morphology of colonies or clusters was not affected by such treatment. However, eosinophil colony formation was inhibited by RA and enhanced by indomethacin.

PGE is supposed to inhibit the formation of monocytes/macrophages by decreasing the sensitivity for CSA of stem cells committed for monocyte production (111). The finding of a reduced granulocyte formation and increased macrophage production by indomethacin in liquid culture is consistent with such an interpretation. The lack of effect of indomethacin in agar cultures may have been due to too low endogenous PGE-production.

Normal and CML colony forming cells were also compared by culture in plasma clots in diffusion chambers placed intraperitoneally in mice. The colony forming cell in DC, CFU-D, is different from the CFU-GM in agar with respect to physical and kinetic properties (39, 46, 47). The number of CFU-D in blood was found to be increased in untreated CML, again emphasizing the expansion of the stem cell pool in this disorder (25, 28, 60, 225-229, 232-235). Chemotherapy-treated patients with nearly normal WBC counts had a normal number of CFU-D in peripheral blood. The colony morphology varied; both macrophage and granulocyte colonies were seen both in normal and CML cell cultures. In analogy with a heterogeneous colony morphology, the density distribution of the CFU-D in Percoll gradients was also heterogeneous. Macrophage colony forming cells predominated among high density fractions, while granulocyte colony forming cells were most abundant in the low density fractions. This discrepancy suggested that the DC-colonies derived from high density fractions were not all progenies of stem cells. As a comparison, colony-forming cells in agar (CFU-GM) are all derived from cells with a density below 1.065 g/ml (61). When DC-colonies derived from cells in the density range 1.068-1.075 g/ml were studied in cell preparations separated according to the presence of Fc-receptors, we found that the Fc-receptor positive cell population also had colony forming cells.

Within the myeloid series Fc-receptors become more abundant with increasing maturity and monocytes also have Fc-receptors (265-267). The fact that more than half of the CFU-D in the high density region of Percoll gradients had Fc-receptors indicate that a considerable amount of these high density CFU-D are cells with intermediate maturity and should not be regarded as true stem cells, and possibly some of them are actually monocytes which proliferate in DC to form small colonies.

A capacity for proliferation (self-renewal) is one of the fundamental properties of stem cells. Self-renewal has been demonstrated for pluripotent stem cells and for blast progenitor cells in AML (18, 29, 268, 269). We observed that the number of normal CFU-GM in suspension culture regularly increased during the first days in culture, whereas the number of CFU-GM in CML consistently failed to show such an increase. This observation suggested that the pre-CFU-GM of normal but not of CML were capable of producing new CFU-GM. Alternatively there is a difference in self-renewal capacity with a higher self-renewal for normal than for CML (CFU-GM).

We approached this problem by recloning of colonies in methyl cellulose and found that normal CFU-GM expressed self-replication, but that colonies derived from CML cells had a defective recloning capacity. The defective self-replication in CML was not related to WBC, duration of the disease or previous chemotherapy.

There are at least two separable populations of CFU-GM, called CFU-GM day 7 and day 14, and evidence has been presented that suggest that CFU-GM day 14 is more primitive than CFU-GM day 7 and actually may be the immediate progenitor for CFU-GM day 7 (37). One conclusion of this evidence is that colonies derived from CFU-GM day 14 may contain colony forming cells but colonies derived from CFU-GM day 7 would not contain any colony forming cells.

The difference in recloning ability between normal and CML cells could thus be due to enrichment in CML of relatively mature colony-forming cells (i.e. CFU-GM day 7) with an inherently low capacity for self-renewal. The findings of a near normal CFU-GM day 7: CFU-GM day 14 -ratio in CML did, however, not indicate that CML cell cultures were enriched for mature colony forming cells. Moreover, when CFU-GM day 7 and day 14 were separated by velocity sedimentation the recloning capacity was confined to fractions containing CFU-GM day 14 both for normals and for CML. Therefore, the data suggest that a deficient recloning ability is a property inherent to CFU-GM of CML. It is still unproven that a defective self-replication is present also in multipotent stem cells.

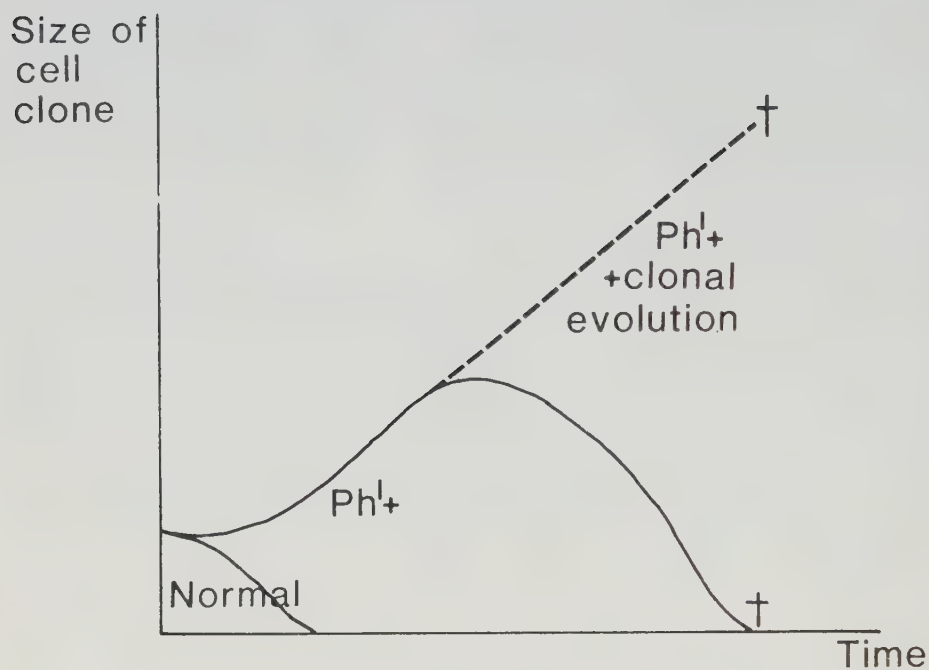


Figure 3. Hypothetical model of the development of cell clones in CML.

Results from experiments with long-term culture of marrow cells from CML patients showed, that leukemia Ph¹-positive cells disappeared whereas karyo-typically normal cells survived for periods of two to three months (221). These data are consistent with our finding of a decreased self-renewal of CFU-GM in CML.

Data obtained from in vitro experiments cannot directly be extrapolated to the patient. However, the possibility of a defective self-renewal capacity of at least granulopoietic stem cells in CML offers a new theoretical model for the explanation of the granulopoietic hyperplasia seen in CML. Some data suggest that the stem cell compartment is structured based on the generation age of the stem cells (7). The model implies that with each cell division the probability for self-renewal decreases and the possibility for terminal differentiation increases. The increased production of mature granulocytes in CML could thus be a consequence of altered probabilities for self-replication and differentiation among granulopoietic stem cells, and the increased concentrations of committed stem cells in CML could theoretically be a consequence of an increased influx from the multipotent stem cell pool rather than an effect of increased self-replication. A hypothetical model of CML is outlined in fig 3. Initially, the normal and leukemic cells coexist, but because of a proliferative advantage due to the increased numbers of proliferating stem cells and to defective inhibition of proliferation and to inhibition of normal granulopoiesis, the leukemic cells become predominating. Eventually, the normal cell clones vanish because of sustained inhibition. The genetic instability inherent to the Ph¹ positive cells will in some instances lead to the development of a blastic crisis, and in other patients a defective self-renewal of the stem cells may result in a termination of the disease in marrow insufficiency.

Fig. 4 summarizes previous and present findings on the granulopoiesis in CML. The number of CFU-GM is greatly increased (60, 234, 235), and a defective self-renewal of CFU-GM may lead to a premature differentiation to mature PMN. Granulocyte progenitors may have abnormally low requirements for growth stimulators.

Moreover, the inhibition of proliferation of granulocyte progenitors in CML by agents such as acidic isoferritins (114, 136, 137), LAI (133, 134), lactoferrin (240-242), PGE (243-245) and T-lymphocytes (246) is defective. Thus several factors seem to contribute to the increased production of granulocytes in CML.

The case report of paper VI suggests that RA may be effective in vivo to induce differentiation in patients with APL. During relapse of the disease a maturation arrest of the marrow promyelocytes was evident in spite of cytostatic

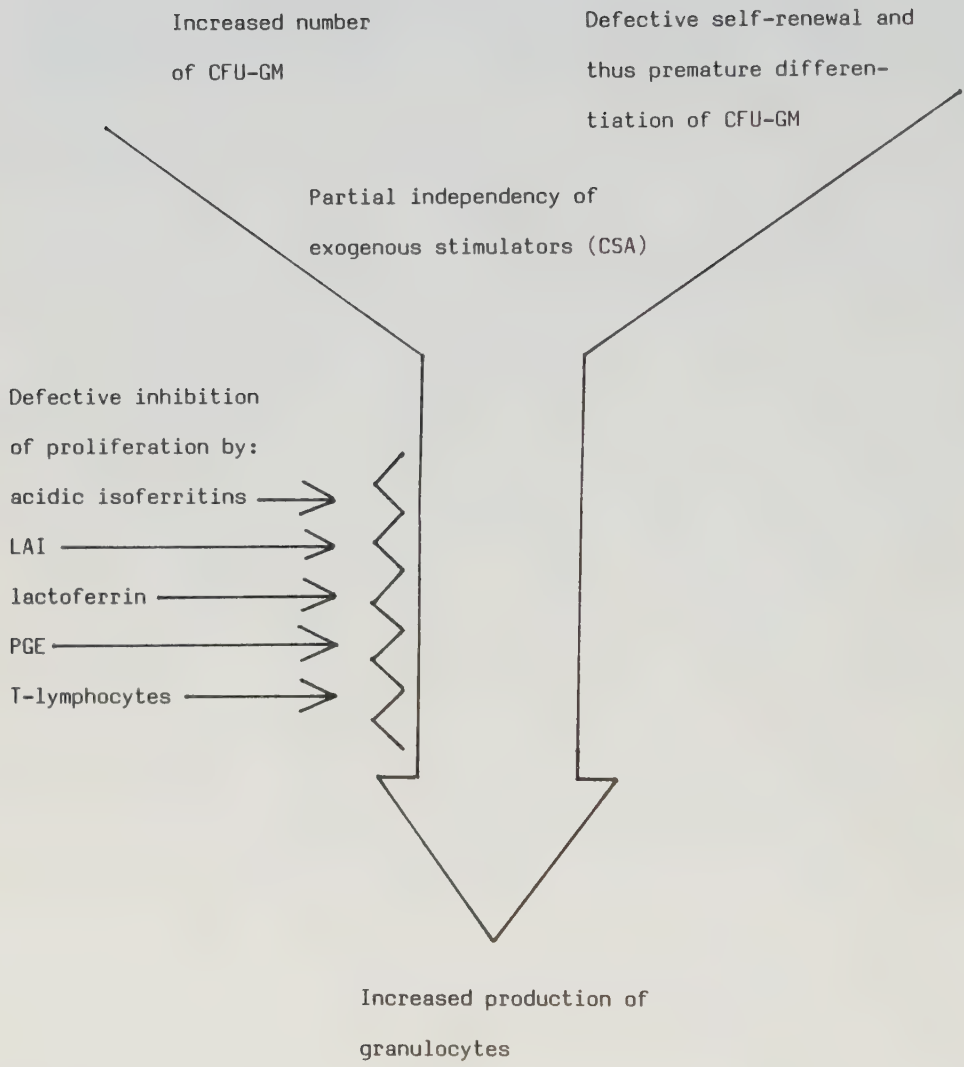


Fig. 4. Model of the granulopoiesis in CML.

treatment. With a temporal correlation to the addition of RA to the previous treatment, this maturation arrest was successively overcome, and the patient entered a complete remission, which has persisted for more than 24 months.

Different features of the patient may be called in question. At presentation, the marrow cells exhibited the typical 15;17 translocation, but this abnormality could not be demonstrated when RA was to be given. Therefore, it was not possible to decide whether the accumulation of maturation-blocked promyelocytes were leukemic or normal. The normal karyotype found at this occasion may reflect coexisting normal cells, or the karyotype of the leukemic cells changed by the chemotherapy or by clonal evolution. It is possible, although unlikely, that the disappearance of the promyelocytes was spontaneous and thus part of the natural course of the disease, or that chemotherapy caused the complete remission. The administration of RA had been preceded by a substantial observation period of about nine months without any major changes in the chemotherapy. Therefore, RA might have been responsible for the effects observed.

The fact that the disappearance of immature cells was not evident until after several weeks of treatment with RA may have several explanations: RA was not responsible for the effect, the effect was indirect or the dose of RA was not optimal. It is, however, also possible that RA alone was responsible for the effect in a direct manner.

The mechanisms for induction of differentiation by RA are not understood. Cell division does not seem to be a requirement for the effect on APL cells in vitro (270). The proliferation of normal marrow granulopoietic cells in culture seems, however, to be enhanced directly or indirectly by RA, probably with the effect limited to the myeloblast-promyelocyte level, as was suggested by the results of paper III.

SUMMARY

This thesis has provided new information on proliferation and differentiation of granulopoietic precursor cells in CML within the following areas:

One class of stem cells, CFU-D, was shown to be expanded in CML as evidenced by an increased number of CFU-D in peripheral blood concomitant with an expanded leukocyte mass. This information confirms and expands earlier observations of increased numbers of stem cells in CML. With regard to density distribution of CFU-D in Percoll gradients no significant deviations from the normal were seen.

Another class of stem cells, CFU-GM, was shown to be defective in its recloning ability, a trait which is present from the time of diagnosis and independent of WBC and previous treatment. This defect is interpreted to imply altered probabilities for self-renewal and differentiation among stem cells in CML, which in effect would lead to an increased influx of committed stem cells and a premature maturation of their progenies.

In line with this reasoning we found that isolated immature CML cells express a higher than normal granulocyte production in DC or suspension cultures. CML cells were partially independent of exogenous growth stimulators and did not show a normal inhibitory response to increasing cell concentrations and both these properties contributed to the increased granulocyte production. The cell production capacity was different for marrow, blood and spleen cells, with the highest efficiency for marrow cells and the lowest for spleen cells. The maturation patterns were similar, however, and also myeloblasts from CML patients in blastic crisis were shown to produce mature-appearing granulocytes during culture in DC.

We found no evidence for an altered commitment of bipotential CFU-GM after treatment with RA or indomethacin, but RA stimulated the formation of granulocytes in liquid cultures probably due to an effect on granulopoietic precursor cells of intermediate maturity as evidenced by the stimulation of cluster formation in agar cultures. Macrophage production was inhibited by RA. Incubation with indomethacin on the other hand, resulted in an increased production of macrophages and a decreased granulocyte production in both CML and normals.

A patient with APL in relapse is described, where the chemotherapy was combined with RA in order to induce differentiation in the maturation-arrested promyelocytes. The successful outcome suggested the possibility of differentiation induction in vivo in at least some patients with AML.

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Proliferation and Differentiation in Diffusion Chambers of Marrow, Blood and Spleen Cells from Patients with Chronic Myeloid Leukaemia during Chronic Phase and Blastic Transformation

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The proliferative and differentiating capacity was compared between immature normal marrow cells and marrow, blood or spleen cells from patients with chronic myeloid leukaemia (CML) in chronic and blastic phases. Low density cells highly enriched in myeloblasts and promyelocytes were cultured in diffusion chambers (DC) and implanted into the peritoneal cavity of mice pretreated with cyclophosphamide. In CML of chronic phase cell production was higher than normal with the exception of spleen cells. Total cell production was lower in blastic phase than in CML chronic phase. The [^3H]-TdR labelling index of myeloblasts of blastic phase CML increased considerably upon implantation in DC consistent with re-entry into active proliferation. Immature normal cells give rise mostly to macrophages while the corresponding CML cells of chronic phase give a much higher PMN production with peaks after 12–18 d. Also in CML of blastic crisis the blasts differentiate into mature PMNs, but the maturation time is shortened with peaks of PMNs after 5–10 d. DC cultures of spleen cells from patients with CML showed a less differentiating capacity with a very low PMN production compared to cultures of marrow or blood cells. Our results indicate intrinsic differences in growth and maturation capacity between normal immature granulopoietic cells and cells from patients with CML of chronic phase or blastic crisis. The results may also indicate intrinsic differences between spleen, blood or marrow cells in CML.

Key words: macrophage – myeloblast – [^3H]-thymidine

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Chronic myeloid leukaemia (CML) is characterized by a massive granulopoietic hyperplasia, which is secondary to hyperplasia of the pool of stem cells committed for granulopoiesis (CFU-c) as detected by the agar culture technique. The growth control still operating in CML (Stryckmanns et al 1977) is apparently not efficient enough to control

the cell proliferation. Since the fraction of CFU-c in DNA-synthesis fluctuates around the normal level (Olofsson & Olsson 1976) it is likely that the hyperplasia is due to a loss of inhibition of stem cell proliferation, which might be due to an intrinsic cellular disturbance or abnormal extrinsic growth regulation. The cell cycle characteristics of CFU-c,

however, vary greatly during the course of the disease in any given subject (Olofsson & Olsson 1976) as an expression of regulatory imbalance emphasizing the necessity for sequential assays in individual subjects. The defective inhibition of stem cell proliferation may also explain the metamorphosis into terminal blast cell transformation, which represents a heterogenous condition occurring along the granulopoietic, lymphocytic or megakaryocytic cell lines. As in many other malignancies, the course of the disease is characterized by increasing abnormalities in a step-wise fashion of the stem cells where the end product is a cell lacking capacity for final differentiation.

Evidently the complexity of CML has not decreased with the recent information on cell production of this disease. In addition, differences have been demonstrated between medullary and extramedullary tissues in CML (Brandt & Schnell 1969, Brandt 1973, Mitelman et al 1974, Müller-Bérat et al 1977). The environment of spleen, liver or marrow might govern cell proliferation and maturation differently.

Diffusion chambers (DC) implanted into the peritoneal cavity of irradiated or cyclophosphamide pretreated mice have been used to culture human leukaemic cells (Chikkappa et al 1973, Fauerholdt & Jacobsen 1975, Hoelzer et al 1977, Hoelzer & Flidner 1977). CML cells have been shown to proliferate in diffusion chambers (Chikkappa et al 1973) and leucocyte alkaline phosphatase activity actually reappeared in the cultured cells. By culturing CML cells in diffusion chambers some of the complexity of the *in vivo* condition can be reduced. The host produces the humoral factors and the semi-solid plasma clot inside the diffusion chamber provides the microenvironment for cell proliferation and differentiation. Since

environmental factors are standardized, variations in growth pattern most likely should reflect abnormalities intrinsic to the cells.

In the present work, we have observed the growth and differentiation in diffusion chambers of cell populations highly enriched in myeloblasts and promyelocytes from marrow, blood and spleen of patients with CML in chronic and acute phase.

MATERIAL AND METHODS

Patient selection

Bone marrow was studied in 8 patients with chronic phase CML. All except 1 patient demonstrated the presence of the Ph¹ chromosome. In 4 patients with chronic phase CML a comparison was made between blood and marrow cells. Furthermore, blood cells were studied in 6 patients with CML in blastic crisis. In addition a comparison was made between spleen and marrow or blood cells in 3 patients. All patients had been off treatment for at least 1 month prior to the study. Marrow was obtained from healthy individuals for controls.

Collection of cells

Marrow, 2–3 ml, was aspirated by sternal puncture and collected in a sterile tube containing 3 ml McCoy's medium with 100 IU of heparin and 75 units of Varidase (Lederle Laboratories, Pearl River, N.Y.). Spleen cells were aspirated in a similar manner at splenectomy. Aspirated particles were disrupted by repeated suction through a needle. Heparinized blood contained 20 IU heparin/ml.

Cell separation

Cells were isolated using density cut centrifugation. Blood and marrow suspensions were mixed with 2 parts of 3% Dextran 250 (Pharmacia, Uppsala, Sweden) in 0.15 M sodium chloride and allowed to stand for 45–60 min at room temp. The leucocyte-rich plasma was withdrawn and the cells collected by centrifugation at $150 \times g$ for 8 min. Density cut separation was performed on isoosmolar Ficoll-Isopaque in balanced salt solution according to Wells et al (1977). The leucocytes were resuspended at a concentration of 10^7 cells/ml in an isoosmolar Ficoll solution with a density of 1.046 g/ml. 10 ml of cell suspension were layered on top of 10

ml of isoosmolar Ficoll-Isopaque with a density of 1.062 or 1.065 g/ml in 50 ml sterile plastic tubes. After centrifugation with a swing-out rotor at $400 \times g$ for 30 min, the cell-rich interphase was withdrawn and the cells washed twice in McCoy's medium with 5% fetal calf serum. Previous studies on continuous isoosmolar Ficoll-Isopaque gradients according to Wells et al (1977) indicated that a density cut separation at 1.062 g/ml should produce cell suspensions highly enriched for myeloblasts and promyelocytes. This was one of the major objectives of the study, to compare the growth in DC of normal and CML myeloblast enriched cell populations.

Diffusion chamber cultures

The diffusion chamber technique was carried out as previously described (Benestad 1970, Bøyum & Borgström 1970). The chambers have a vol of 140 μ l and consist of a plastic ring (Millipore PR 0001401) with a 0.22 μ m porosity filter (Millipore GSWP01300) glued to each side of it with MF cement (Millipore XX 70 000 00). The chambers were tested for leaks and sterilized for 2–4 h in UV-light. Each chamber was filled with 100 μ l cell suspension in McCoy's medium with 5% fetal calf serum and sealed with a plastic plug, which was melted to the (plastic) ring. The chambers were kept in culture medium before implantation. The cell inoculum number was $2.5\text{--}4 \times 10^5$ nucleated cells per DC. The rate of cell production was unchanged when the initial cell concentration was decreased. Thus, the results obtained cannot be explained by overloading the DC culture system. The differential counts of the cell inoculum are shown in Figures 2, 3, 5 and 6.

Male NMRI mice, weighing 22–34 g were used for implantation. 6–24 h before implantation, cyclophosphamide, 275 mg/kg, was injected intraperitoneally. During pentobarbital anaesthesia, 2 chambers were implanted intraperitoneally into each mouse, followed by instillation of a 1% penicillin-streptomycin solution.

4–8 chambers were harvested 2–3 times a week for 3–4 weeks. The chambers were agitated in 0.5% Pronase (Sigma) in 5% Ficoll (Pharmacia, Sweden) in McCoy's medium at room temp. for 1 h. Thereafter, they were punctured, the contents withdrawn, and washed out with McCoy's medium. The cells were counted and the content from 4–8 chambers pooled. Smears were stained with May-Grünwald Giemsa and differential counts were made on at least 400 cells. The rest of the cell suspension was washed twice in McCoy's medium with 5% fetal calf serum and cultured in agar.

In vivo [^3H]-TdR labelling of cells during DC culture

25 μ Ci of [^3H]-TdR (specific activity 7 Ci/mmol, Radiochemical Centre, Amersham, England) was injected intraperitoneally 1 h before harvesting the chambers. The [^3H]-TdR labelling indices were determined by autoradiography using emulsion K2 (Ilford Ltd., England).

Agar culture technique

The culture technique of Pike & Robinson (1970) was utilized. The feeder layers contained 3×10^5 mononuclear blood cells from a healthy donor in 1 ml of 0.5% agar (Difco Bacto Agar) in McCoy's medium plated in 35 mm Falcon plastic petri dishes. The cover layers contained 10^5 freshly isolated cells or cells harvested from diffusion chambers plated in 1 ml 0.3% agar in McCoy's medium. After gelling at room temp. the plates were incubated at 37°C in a fully humidified atmosphere with 7.5% CO_2 in air. Cultures were scored on d 10 for large clusters (20–40 cells) and colonies (> 40 cells).

Statistical analysis

The mean values of the total cell counts per diffusion chamber were calculated together with standard error of the mean (SEM). The amount of each cell type per diffusion chamber was assessed using total cell count, differential count and the SEM.

RESULTS

The cell separation procedure adopted consisted of density cut centrifugation at a density of 1.062 g/ml (except for the comparison between CML blood and marrow cells where a density of 1.065 g/ml was used) to obtain a low density cell fraction dominated by immature myeloid cells. For normal marrow cells the recovery of CFU-c in the cell fraction with a density lower than 1.062 g/ml was 75 ± 6 (SD) % (4 experiments). Judging from centrifugation in continuous density gradients according to Wells et al (1977) the peak density for normal CFU-c varied between 1.057–1.062 g/ml (4 experiments). Similar experiments with CML cells showed a considerable variation for the peak density

of CFU-c; it was generally below 1.062 g/ml. The conclusion is that variations in recovery of CFU-c does not explain differences presented below for maturation in diffusion chambers of normal and CML low density marrow cells.

Total nucleated cells

Figure 1 gives the change in total nucleated cells per chamber with duration of DC culture for normal controls and the patients studied during chronic phase or blastic transformation of CML. All the experiments depicted in Figure 1 were with cells obtained

by density cut centrifugation at 1.062 g/ml. In chronic phase CML marrow cells were used while blood cells were used for culture at blastic transformation due to the difficulties in aspirating marrow cells at the latter stage of the disease. Normal controls showed an initial decrease in total cell counts followed by a peak after 11–14 d. In chronic phase CML the marrow cells in 5 of 8 patients showed an initial decrease in total nucleated cells for 2–3 d of culture, followed by a variable increase to a maximum at d 7–12 (5 of 8 patients); 2 of 8 patients showed a peak after 16–18 d in DC and 1 pa-

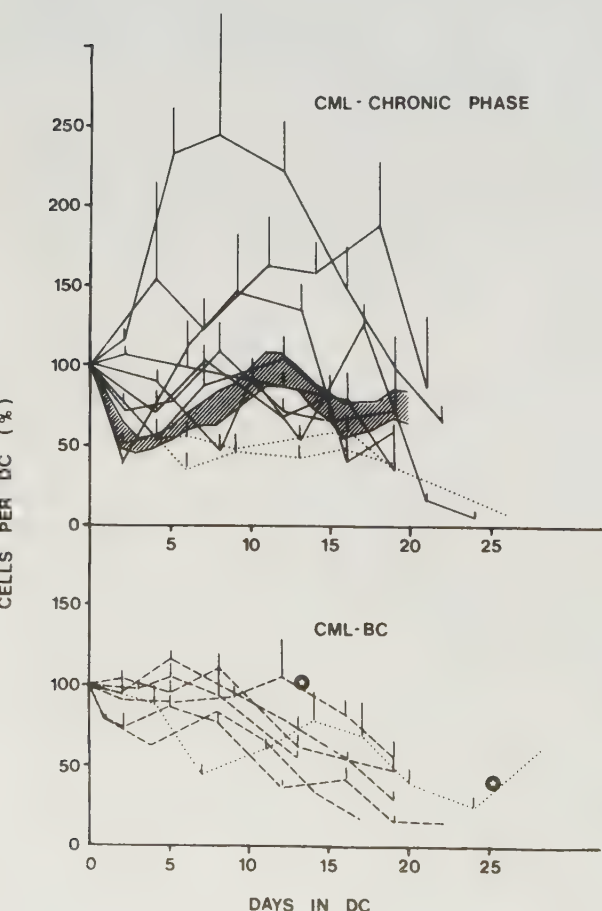


Figure 1. Change in number of total nucleated cells per chamber with duration of DC culture in CML-chronic phase and CML-blastic crisis (BC) and normal controls. CML marrow cells (—), normal marrow cells (hatched area), blood cells (---) and spleen cells (...). Blood and spleen cells of the patient in blastic crisis where also spleen cells were cultured are indicated by asterisk. The SEM is given as a vertical bar.

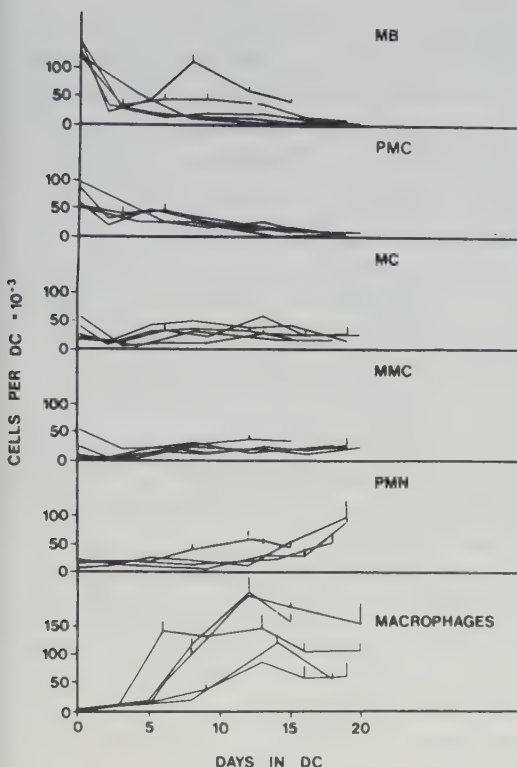


Figure 2. Change in number of myeloblasts (MB), promyelocytes (PMC), myelocytes (MC), metamyelocytes (MMC), polymorphonuclear cells (PMN), and macrophages upon DC culture of normal marrow cells.

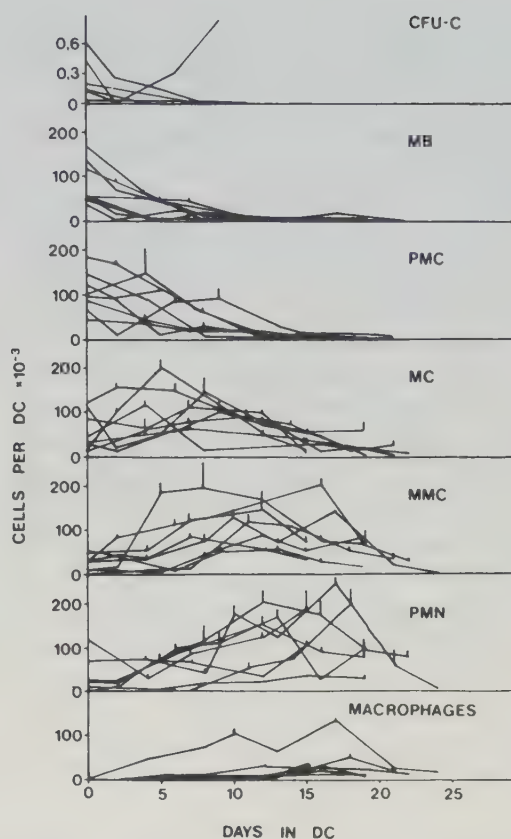


Figure 3. Change in number of CFU-c, myeloblasts (MB), promyelocytes (PMC), myelocytes (MC), metamyelocytes (MMC), polymorphonuclear cells (PMN) and macrophages upon DC culture of marrow cells in CML-chronic phase. The SEM is given as a vertical bar.

tient showed no peak. Spleen cells produced distinctly lower numbers of nucleated cells than marrow cells. Blood cells from patients with CML in blastic transformation showed no clear-cut increase with culture; the cellularity of the DC decreased after 8 d. Spleen cells obtained from 1 patient in blastic crisis produced a lower amount of nucleated cells than blood cells from the same patient. The total production in DC was higher in CML of chronic phase than in normals except for spleen cells, where production was lower in blastic crisis as compared to chronic phase CML.

Differentiation of normal cells

Figure 2 depicts the differentiation pattern of normal marrow cells with a density below 1.062 g/ml. The differential counts were comparable to those of CML in chronic phase with predominantly myeloblasts and promyelocytes. Recovery of such cells was about 60%. In DC the normal cells produced very low numbers of mature granulocytes, the rise at the end of the cultures consisted mostly of eosinophils. Macro-

phage production was, however, prominent with a maturation time of 12–14 d.

Differentiation of chronic phase CML cells

Figure 3 shows the maturation of CML marrow cells obtained by density cut centrifugation at 1.062 g/ml. A relatively uniform cell population was obtained consisting of 25–40% myeloblasts and 30–50% promyelocytes. These cell populations contained approximately 20–70% of the myeloblasts and 10–20% of the promyelocytes of the initial marrow aspirate obtained from the patients. Due to the dominance of the most immature cells at initiation of DC culture it is possibly to get reliable data on the maturation of CML cells in DC.

CFU-c, initially present in varying

amounts, showed a marked decrease with time in most patients with disappearance after 4–12 d; an increase with time was seen in 1 patient. Also myeloblasts showed a marked initial reduction with a slight secondary increase after 8–18 d. A similar pattern was seen for promyelocytes but in some samples slight early elevations were noted but also low secondary peaks on d 8–18. Myelocytes showed an increase with a peak on d 5–11 and then disappeared. Metamyelocytes, almost completely absent initially, peaked on d 8–17 and thereafter disappeared. Mature granulocytes were produced with a peak after 12–18 d in DC. 1 patient, however, displayed a maturation pattern with formation of very few mature granulocytes. The latter findings was also typical for spleen cells (see below). Mature

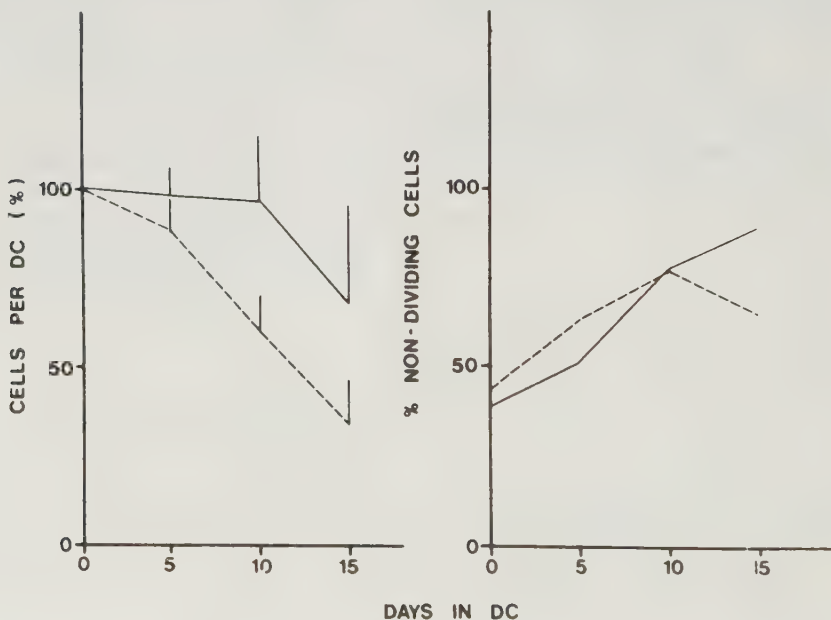


Figure 4. Comparison in four patients of changes in number of total nucleated cells per chamber and % non-dividing cells (metamyelocytes, mature granulocytes and macrophages) with duration of DC culture for blood (---) and marrow (—) cells in CML chronic phase. The SEM is given as a vertical bar.

eosinophils showed a peak at later time than neutrophils (results not shown). Macrophages started to appear after 1 week in DC and generally increased slowly with time.

It was noted that the production of mature granulocytes was much higher in CML of chronic phase as compared to normal marrow cells (Figure 2). On the other hand, production of macrophages was lower in CML compared with normal marrow cells.

Comparison between CML blood and marrow cells

For a comparison between DC growth of blood and marrow in chronic phase CML, cells were isolated from 4 patients by density cut separation at a density of 1.065 g/ml. DC were analyzed at 5, 10 and 15 d of culture (Figure 4). The total production of nucleated cells per chamber was lower for blood as compared to marrow, but the fraction of non-dividing cells did not differ.

Differentiation of CML cells in blastic crisis

The cell suspensions isolated from blood by density cut separation at a density of 1.062 g/ml were highly dominated by blasts (Figure 5). CFU-c decreased and disappeared after 5–10 d of culture. A substantial number of the myeloblasts disappeared during the first 5 d of culture judging from the appearance of degenerated and vacuolated blasts. A part of the blasts, however, obviously proliferated and gave rise to cell maturation. Cell proliferation is also indicated by the findings from investigation of the actual number of blasts in DNA synthesis by [^3H]-TdR labelling in vivo by intraperitoneal injection 1 h before harvesting the chambers (Table 1). A profound increase of labelling indices of blast cells was found after 2 d in DC but a decrease was seen after

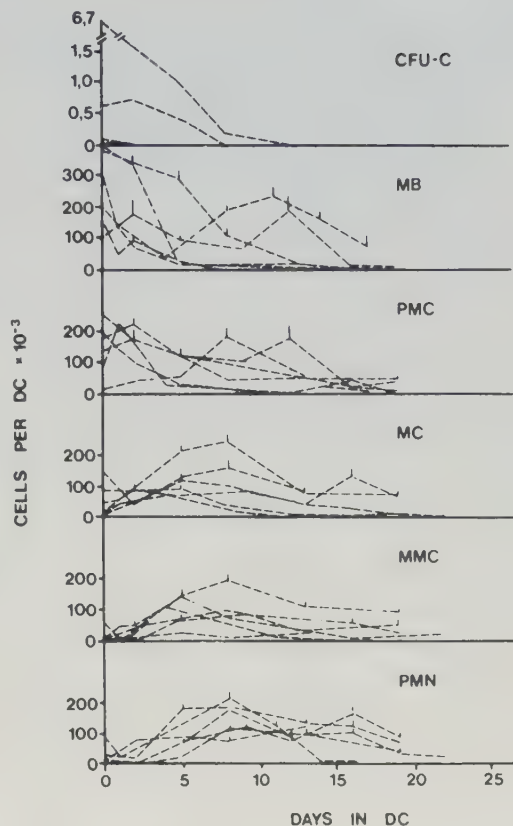


Figure 5. Change in number of CFU-c, myeloblasts (M.B), promyelocytes (PMC), myelocytes (MC), meta-myelocytes (MMC) and polymorphonuclear cells (PMN) upon DC culture of blood cells in CML-blastic crisis.

5 d. The maturation into promyelocytes and myelocytes was actually very similar to that of marrow cells obtained during chronic phase CML (Figure 3). But peaks of meta-myelocytes and mature granulocytes appeared already after 5–10 d indicating premature production of mature looking cells during blastic crisis. However, the total production of metamyelocytes and mature granulocytes was lower than during chronic phase. In 2 of 6 patients a second peak of blasts was seen on d 10–12. These cells, too,

TABLE 1

In vivo [^3H]-TdR labelling of blast cells from CML blastic crisis during diffusion chamber culture

Patient	^3H -TdR labelling index		
	Day 0	Day 2	Day 5
B.E.	29	56	38
S.R.	4	40	15
A.M.B.	10	25	22

seemed to give rise to some maturation with peaks of myelocytes, metamyelocytes and PMNs around d 16. Macrophages were virtually absent in DC cultures of cells from patients with CML in blastic crisis.

Comparison between spleen, blood or marrow

In 1 patient with chronic phase CML, a comparison was made between DC growth of spleen and marrow cells. In 2 patients, 1 with chronic phase CML and 1 with blastic crisis, a comparison was made between DC growth of spleen and blood cells. Cell separation was performed with density cut centrifugation at a density of 1.062 g/ml. The composition of cells separated from different sources of the the same individual showed some variation. Distinct differences were seen in DC growth between spleen and blood of marrow cells (Figure 6). The spleen cells produced much less maturing cells as compared to blood or marrow and mature granulocytes were scarce in the DC cultures. This conclusion is valid also when the variation of the composition of the cell suspensions obtained from different sources is taken into consideration. DC cultures of spleen cells showed a tendency for persistence of myeloblasts and promyelocytes also after more than 20 d.

DISCUSSION

Previous work on CML cells in diffusion chamber culture has been performed with a mixture of marrow cells (Chikkappa et al 1973). Such studies showed the expected growth rate for neutrophilic cells using the model for normal granulopoiesis suggested by Cronkite & Vincent (1969). The results were confirmed in the present study (results not shown) using CML marrow cells isolated by density cut centrifugation at a density of 1.077 g/ml; the cells obtained represent a mixture of marrow cells except that mature granulocytes had been removed. However, we considered it more important to compare the behavior of normal and CML myeloblasts instead of working with a mixture of granulopoietic cells at various stages of maturation. Such studies have not been reported previously. By centrifugation at a density of 1.062 g/ml a light density fraction was obtained consisting almost exclusively of myeloblasts and promyelocytes. As the recovery and composition of the cell specimens obtained were similar for normal and CML marrow, a comparison of DC growth seemed valid. That the differences observed in growth and maturation capacity of low density normal and CML marrow cells were due to density differences between normal and CML cells capable of giving rise to mature granulocytes in DC is unlikely. Thus, the recovery of CFU-c in the low density cell fraction used for DC culture was similar for normal and CML marrow cells. It is emphasized that the density of haemopoietic cells can vary considerably in different density gradient systems. The peak density for normal CFU-c was 1.064 g/ml using Percoll density gradients (Olofsson et al 1980), which is a considerably higher density compared to the present results from

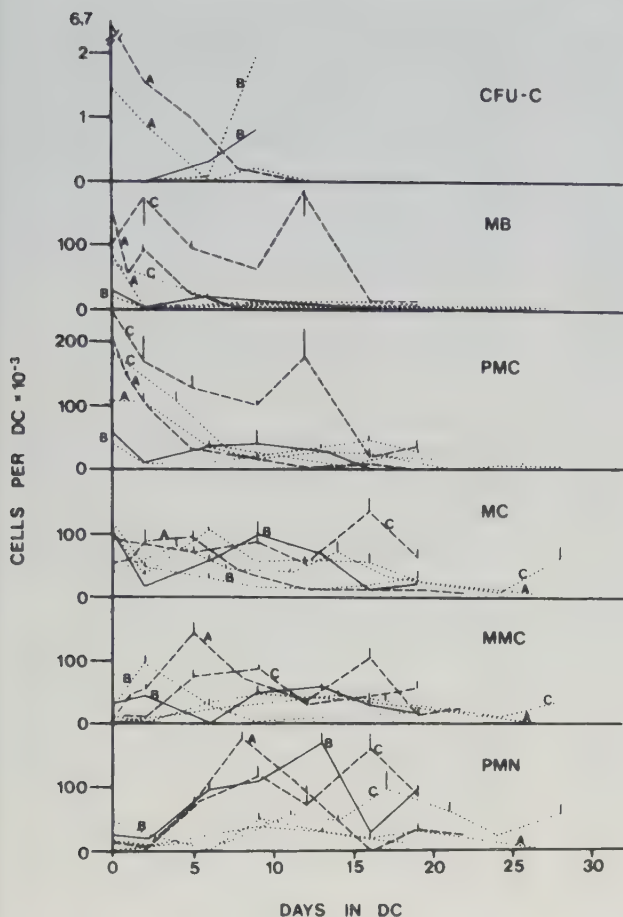


Figure 6. Comparison between DC growth of spleen, blood or marrow cells in CML. Data are given for changes in number of CFU-c, myeloblasts (MB), promyelocytes (PMC), myelocytes (MC), metamyelocytes (MMC) and polymorphonuclear cells (PMN). Spleen cells (---), blood cells (---), and marrow cells (—). Patients A (B.E.) and B (E.J.) represent CML-chronic phase while patient C (M.S.) represents CML-blastic crisis. Patient A showed, however, a hyperdiploid karyotype of spleen cells at the time of the study but not of marrow cells. He developed signs of blast cell transformation of marrow and blood 1 month later.

isoosmolar Ficoll-Hypaque.

It was surprising to find that a mixture of myeloblasts and promyelocytes obtained from normal marrow produced very few mature granulocytes in DC culture in spite of the fact that such specimens contained approximately 60% of the myeloblasts of the original marrow. On the other hand, the production of macrophages was considerable. A possible explanation is that the growth factors necessary for granulocyte production from myeloblasts are lacking in the mice but growth factors necessary for macrophage production are adequately

provided by the host. The observation that the myelocytes of mixed marrow cell suspensions matured into PMNs suggests that maturation is governed differently for myeloblasts and myelocytes.

Contrary to the fraction of normal light density, myeloblasts and promyelocytes of CML marrow matured in an orderly fashion during DC culture producing a peak of mature granulocytes after 12–18 d. However, an even higher initial increase in total nucleated cells as well as a higher PMN production would have been expected on the basis of the model for

normal granulopoiesis (Cronkite & Vincent 1969). The difference between DC growth of immature normal and CML cells, it is suggested, depends on different needs for factors stimulating proliferation and differentiation. Alternatively, such normal and CML cells are at different stages of differentiation in spite of similar morphology and therefore subjected to growth regulation in a non-identical manner. That the proliferation of light density CML cells in DC culture was actually produced by leukaemic cells was confirmed by karyotype analysis (Nilsson et al, to be published).

The present study also showed that myeloblasts from patients with CML in blastic transformation exhibited maturation in an orderly fashion giving rise to successive peaks of promyelocytes. Peaks of metamyelocytes and PMNs occurred almost simultaneously with the peak of myelocytes. The production of PMNs was lower than for chronic phase CML. Even if the myeloblasts of blastic transformation showed a considerable death rate in DC the production of myelocytes was similar to that of chronic phase. Thus, the final maturation in DC culture into PMNs is deficient in CML of blastic crisis. Furthermore, in contrast to chronic phase CML the maturation in DC culture of myeloblasts of blastic crisis was shortened with peaks of PMNs appearing already after 5–10 d. The comparison between chronic phase and blastic crisis CML may not, however, be completely valid since marrow and blood cells, respectively, were used for the DC cultures. The shortened maturation time found in blastic crisis may result in defective cytoplasmic maturation of the mature appearing PMNs supported by the finding of granular abnormalities in PMNs isolated from patients with CML in blastic crisis (Olofsson et al 1976). That pro-

liferation actually occurred in clones responsible for metamorphosis into blastic crisis with chromosome abnormalities in addition to the Ph¹-chromosome was confirmed by karyotype analysis (Nilsson et al, to be published).

The considerable increase in the [³H]-TdR labelling index of blasts obtained from patients in blastic transformation may indicate re-entry of leukaemic blast cells into active proliferation upon DC culture. But the results are influenced to some extent by initial depletion of the number of blasts in the DC cultures. Similar findings have previously been reported from DC culture of AML blood cells (Hoelzer & Fliedner 1977). In those studies no correlation was found between blast cell growth in DC and the number of implanted leukaemic blast cells in S-phase. Recruitment of resting leukaemic blast cells was postulated. An initial depletion, preferently of non-cycling cells could also occur.

The present finding of a less differentiating capacity of spleen cells as compared to marrow (or blood) cells with low final neutrophil maturation in DC culture indicates intrinsic differences between spleen and marrow cells in CML. There are several previous indications that spleen and marrow cells can behave differently in CML. The composition of spleen and marrow cells (Brandt & Schnell 1969, Müller-Bérat et al 1977) as well as the kinetic properties of these (Brandt 1973) often show differences. Moreover, the abnormal clone preceding the blastic phase has been found in the spleen before it was demonstrated in the marrow (Mitelman et al 1974). Actually, one of the patients of the present study demonstrated the latter phenomenon (Figure 6). But elective splenectomy during CML does not result in a detectable differ-

ence in the rate of blastic transformation between splenectomized and non-splenectomized patients (Italian cooperative study group 1978). We suggest that cells with certain abnormalities do not have a proliferative advantage in the marrow of CML patients. They could be selectively retained by the spleen where the environment may be more suitable for proliferation. A combination of both extrinsic environmental abnormalities and intrinsic cellular disturbances might be reflected in the results of DC culture. The stem cell population which eventually has shifted from the marrow to the spleen may produce a progeny with lower capacity for maturation than marrow cells as found in the DC studies. It is unlikely, however, that the spleen has a primary role for the appearance of the increasing abnormalities occurring in a step-wise fashion during the natural course of CML. This process could appear at random anywhere but additional abnormalities could be visible in the spleen because of selective retention of abnormal cell populations.

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Proliferation and differentiation of normal and chronic myeloid leukaemia (CML) marrow cells in suspension cultures

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Immature myeloid cells highly enriched for colony-forming cells, myeloblasts and promyelocytes were isolated in Percoll gradients (lighter than 1.065 g/ml) from patients with chronic myeloid leukaemia (CML) or normal healthy volunteers. The proliferation and differentiation of these cells were followed in suspension cultures in McCoy's medium (15 % FCS) with or without the addition of 15 % human placenta conditioned medium (HP-CM) over a culture period of 12–16 d. Ion-exchange chromatography and gel chromatography indicated that the proliferation-stimulating activity in HP-CM was colony-stimulating activity (CSA). In unstimulated cultures (without HP-CM), normal cells produced very few neutrophils; macrophage production dominated. CML cells, on the other hand, gave rise to neutrophils even in the absence of stimulatory factor(s). The effect of HP-CM was dependent on the initial concentration: at a 'low' (1×10^5 /ml) concentration, the addition of HP-CM resulted in a great increase in neutrophils, but at a 'high' ($5-8 \times 10^5$ /ml) initial cell concentration, HP-CM gave only minimal increase in neutrophil numbers in both normal and CML cultures. These observations suggest endogenous differences between normal and CML precursors in their requirements for or responses to growth stimulatory or inhibitory factors.

Key words: chronic myeloid leukaemia – differentiation – suspension culture

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ABBREVIATIONS

- CML = chronic myeloid leukaemia
- HP-CM = human placenta-conditioned medium
- CFU-GM = colony-forming unit – granulocyte, monocyte/macrophage
- CSA = colony stimulating activity

The most prominent feature of chronic myeloid leukaemia (CML) is the granulocytic hyperplasia associated with an expansion of the stem cell pool committed for

granulopoiesis (1, 2). Abnormalities in growth regulation such as defective feedback regulation (3), decreased activity for prostaglandins (4, 5) and for lactoferrin (6) may also play a rôle in the expansion of the granulocytic cell mass.

Experiments with cells in diffusion chambers implanted in the peritoneal cavity of mice have shown differences in the growth and maturation patterns between normal

and CML cells (7). Thus, immature marrow cells from patients with CML in chronic phase showed a higher cell production than normal. Normal immature marrow cells gave rise mostly to macrophages, where CML cells preferentially produced mature granulocytes. The granulopoietic stem cell, CFU-GM, is potent for the production of both neutrophils and monocytes. The differences in maturation pattern observed for CML and normal marrow cells in diffusion chambers could hypothetically derive from an altered commitment of CML progenitor cells with an amplification of the granulopoietic pathway. The differences observed could, however, also be due to the culture conditions in the diffusion chambers. To get additional information about differences in proliferation and differentiation between normal and CML marrow cells, we have now studied such cells in suspension cultures.

MATERIAL AND METHODS

Collection of bone marrow

Sternal bone marrow was obtained from patients with CML in the chronic phase and from healthy volunteers, and the cells suspended in McCoy's medium containing heparin (100 IU/ml) and Varidase® (75 U/ml; Lederle, Pearl River, NY). An equal vol. of 2% Dextran (T500, Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.15 mol/l NaCl was added and the red cells were left to sediment for 30–45 min at room temp. The leucocyte-rich plasma was collected, centrifuged at $500 \times g$ for 5 min and the cells resuspended in McCoy's medium with 1% fetal calf serum (FCS).

Cell separation

A modification of a previously-described method for separation in Percoll gradients was used (8); a discontinuous gradient consisting of 10 ml Percoll solution with a density of 1.077 g/ml and 10 ml of 1.065 g/ml solution in a 50 ml centrifuge tube (Falcon Plastics, Los

Angeles, Calif.) was cautiously over-layered with 10 ml cell suspension with a maximal cell concentration of 2×10^7 cells/ml. Centrifugation was in a swing-out rotor at $700 \times g$ for 20 min. The cells from the two inter-phases were collected and washed twice in McCoy's medium with 1% FCS. Thus, two cell suspensions were obtained, one with cells lighter than 1.065 g/ml and one with cells with a density of 1.065–1.077 g/ml.

Suspension cultures

Isolated cells were incubated in 1.5 ml aliquots in 12 $\times$ 75 mm plastic tubes (Falcon Plastics) in McCoy's medium with 15% FCS with or without 15% human placenta conditioned medium (HP-CM) prepared as described by Burgess et al (9), at cell concentrations indicated in the results, incubated in 5% CO₂ in a fully humidified atmosphere at 37°C, and harvested 2–3 times a week for 12–16 d. The cells were counted in a haemocytometer and used for preparation of cytocentrifuge smears and for CFU-GM colony assays in agar. Smears were stained with May-Grünwald-Giemsa and 400 cells counted for differential morphology.

Assay of CFU-GM formation in agar

On day 0 and on each subsequent harvest of cells from the suspension cultures, an aliquot of the cells was cultured in agar to measure the number of CFU-GM per culture. The cells, not exceeding 1.5×10^5 per dish, were immobilized in 0.3% agar (Bacto-Agar, Difco) in 1 ml McCoy's medium containing 15% HP-CM as source of colony stimulating factors (CSF) on top of 1 ml 0.5% agar in 35 mm petri dishes (Falcon Plastics). Four replicates were made and the dishes were incubated as described for suspension cultures. Colonies of more than 40 cells were counted in an inverted microscope on days 7 and 14 of incubation.

Ion-exchange chromatography and gel chromatography of HP-CM

Ion-exchange chromatography on DEAE-Sepharose and gel chromatography on Sephadex G-75 of HP-CM was performed as described previously (10). Each fraction of the chromatograms was incubated at 10% concentration in suspension cultures (1×10^5 cells/ml lighter than 1.065 g/ml) and in agar cultures to detect stimulatory activities for cell-production and colony-stimulating activity (CSA), respectively. Suspension cultures were harvested on day 9 and colonies counted on day 10.

RESULTS

Cellular composition of isolated cells

Figure 1 shows the composition of normal and CML bone marrow cells isolated in Percoll at 1.065 and 1.065–1.077 g/ml. Virtually all colony forming cells were covered within the fraction lighter than 1.065 g/ml. As expected, the concentration of CFU-GM was increased in CML compared to normal both for day 7 and day 14. Early granulopoietic precursor cells, i.e. myeloblasts and promyelocytes, were highly concentrated in the light density fractions and together made up more than 50% of all cells in both CML and normals; in this respect, the starting cell suspensions were very similar for CML and normals. The main difference in cell composition in the light density fractions between CML and normals was the amount of lymphocytes,

which was on the average 4 times higher in normals (30% vs 7.5%). The cells within the density range 1.065–1.077 g/ml were mainly late granulopoietic cells with myelocytes, metamyelocytes and PMN in increasing concentrations. Also in this fraction there was a higher proportion of lymphocytes in the normals.

Total cell production in suspension cultures

Preliminary experiments indicated that the total cell production was dependent on the initial cell concentration and that the stimulatory effect of HP-CM was evident only when the cell concentration was reduced. Figure 2 shows that at a starting cell concentration of approximately $8.1 \times 10^5/\text{ml}$ for normals and $4.7 \times 10^5/\text{ml}$ for CML with cells lighter than 1.065 g/ml, total cell production was insignificant for normals and reached only 1.5–2.0 times the initial concentration in the CML cell suspensions.

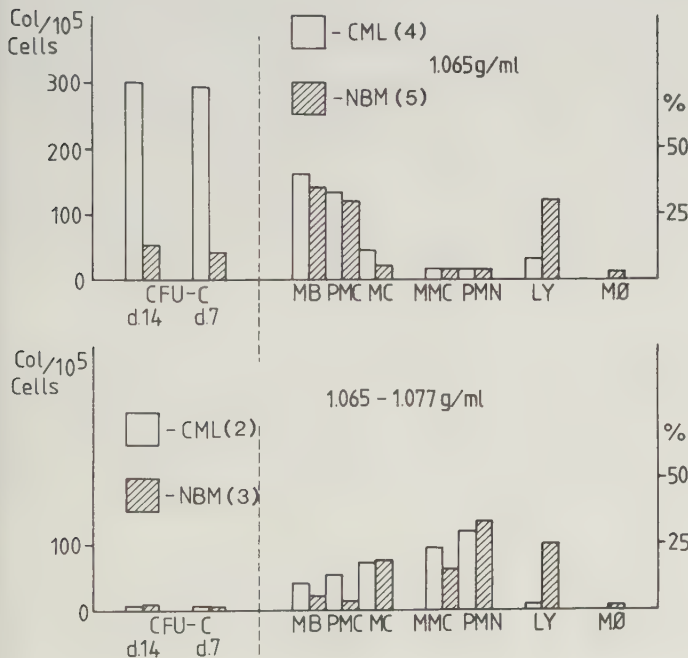


Figure 1. Composition of normal and CML bone marrow cells after separation in Percoll at 1.065 g/ml (upper panel) and 1.065–1.077 g/ml (lower panel). The left part shows the number of colony-forming cells on days 7 and 14.

The right-hand part shows the % of myeloblasts (MB), promyelocytes (PMC), myelocytes (MC), metamyelocytes (MMC), mature neutrophils (PMN), lymphocytes (LY) and monocyte-macrophages (MØ).

The numbers given are mean values of the number of patients tested given in brackets.

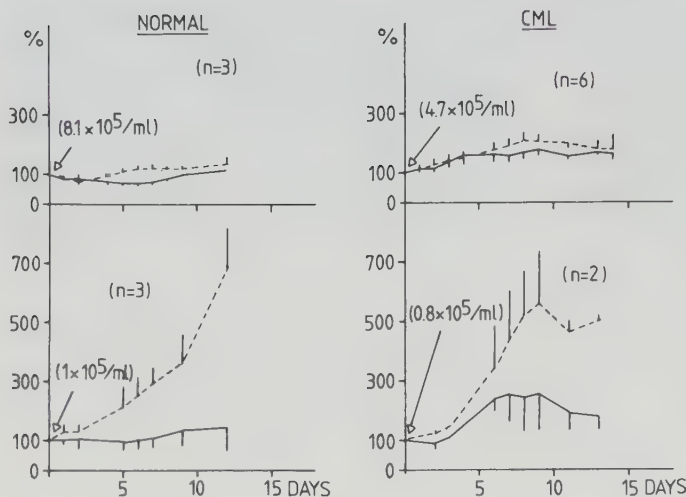


Figure 2. Change of the number of total nucleated cells in suspension cultures of normal (left) and CML (right) cells lighter than 1.065 g/ml at the mean starting concentrations shown in the figure.

(—), control without HP-CM; (---), with HP-CM added. Vertical bars show SEM.

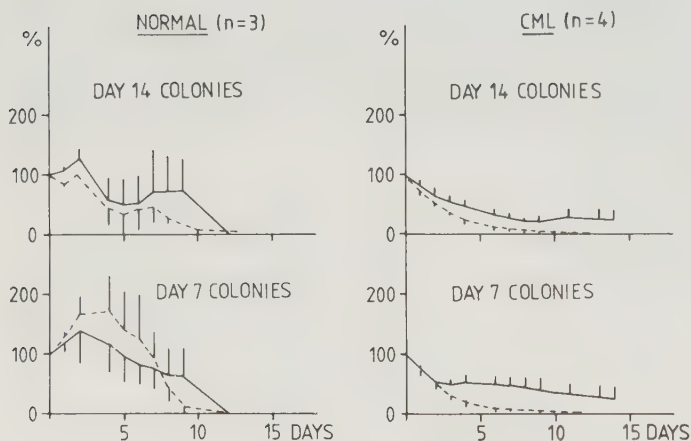


Figure 3. Change in the number of colony-forming cells (CFU-GM day 14 and day 7) in suspension cultures of normal and CML bone marrow cells lighter than 1.065 g/ml.

On day 0, normal cell cultures had 8.1×10^5 cells/ml (mean) and 426 ± 252 CFU-GM day 14 (= 100 %) and 322 ± 40 CFU-GM day 7 (= 100 %). The CML cell cultures were started with 5.2×10^5 cells/ml and 1555 ± 343 CFU-GM day 14 and 1513 ± 257 CFU-GM day 7.

(—), control cultures; (---), cultures with HP-CM added. Vertical bars show SEM.

Addition of HP-CM had a minimal if any effect at these cell concentrations.

On the other hand, if the starting cell concentration was reduced to $0.8\text{--}1.0 \times 10^5$ /ml (Figure 2, lower panel) (cells lighter than 1.065 g/ml) the addition of HP-CM had striking effects. Normal cells without the addition of HP-CM did not produce a net increase over the 12-day culture period, but

with HP-CM supplementation, there was a more than 6-fold increase in cell concentration. CML cells increased about 2-fold even without HP-CM, but reached a 5-fold increment in the presence of HP-CM.

CFU-GM in suspension cultures

The number of early (day 14) and late (day 7) CFU-GM were followed over a 12-day

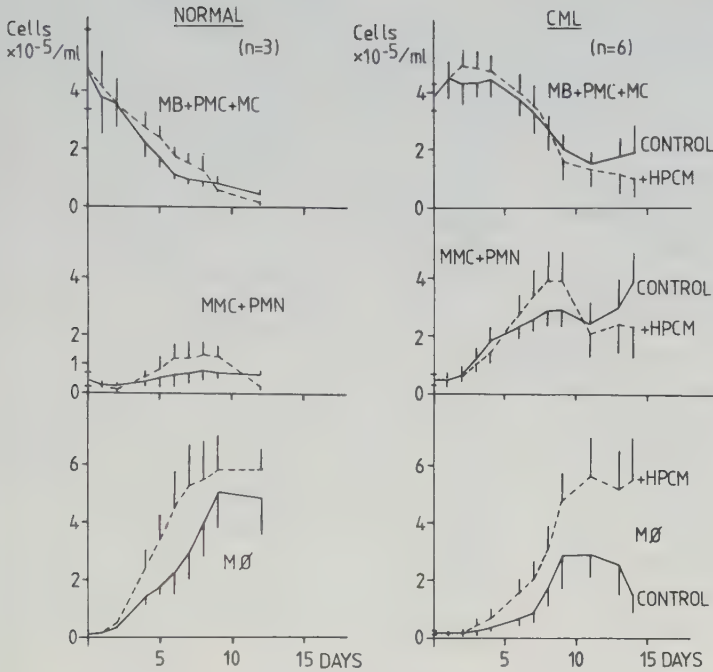


Figure 4. Growth of light density (<1.065 g/ml) cells in suspension cultures.

Starting cell concentrations (total cells) were 8.1×10^5 /ml (normals) and 4.7×10^5 /ml (CML).

Abbreviations as in Figure 1. Vertical bars show SEM.

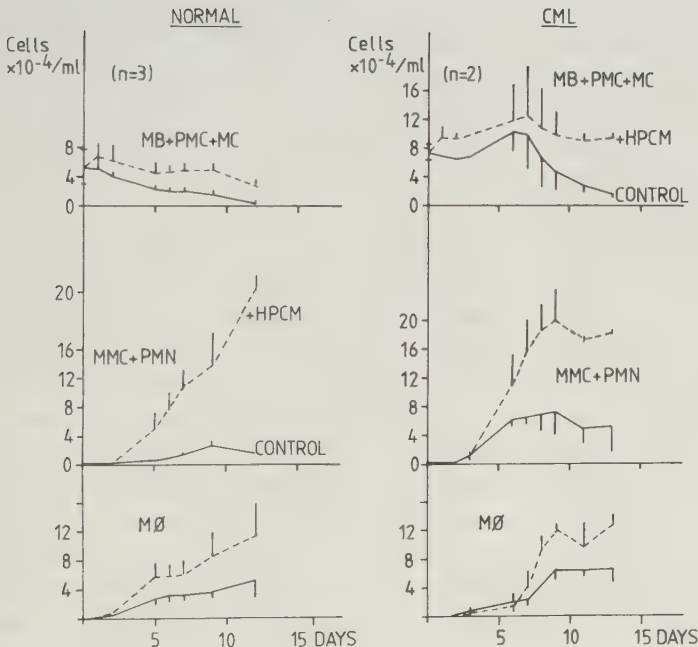


Figure 5. Growth of light density (<1.065 g/ml) cells in suspension cultures at starting cell concentrations 1.0×10^5 cells/ml (normals) and 0.8×10^5 cells/ml (CML). Abbreviations and symbols as in Figure 4.

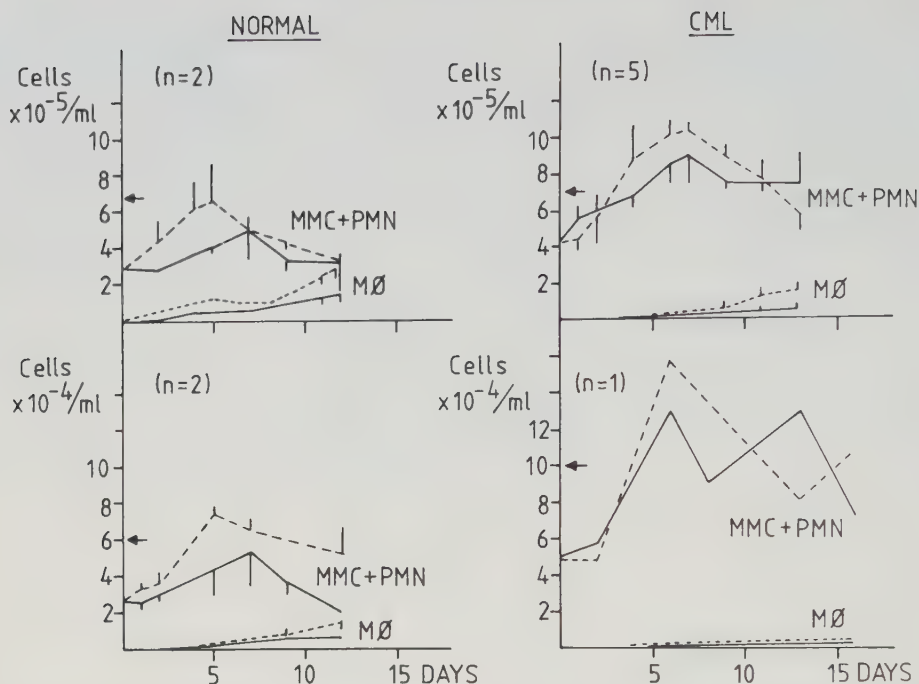


Figure 6. Production of neutrophils (MMC + PMN) and macrophages (MØ) in suspension cultures from medium density (1.065–1.077 g/ml) normal and CML bone marrow cells. Starting cell concentrations are indicated by the arrows. (—), control without HP-CM; (---), HP-CM added.

culture period at the starting cell concentrations $8.1 \times 10^5/\text{ml}$ (normals) and $4.7 \times 10^5/\text{ml}$ (CML). Figure 3 shows that in CML, both CFU-GM day 14 and day 7 steadily decreased over the culture period; in the case of CFU-GM day 7, the addition of HP-CM accelerated the decline in colony forming cells. With normal bone marrow cells (Figure 3, left part), CFU-GM day 14 increased slightly during the first 2 d of culture but then decreased to zero until day 12; HP-CM had minimal effect on these cells. Normal CFU-GM day 7, on the other hand, showed a significant increase over the first 2–4 d of culture in the presence of HP-CM and thus resulted in a higher production of CFU-GM day 7 during days 2–6 of culture

in comparison with the control cultures without added HP-CM. These experiments show unequivocal differences in the behaviour of normal and CML CFU-GM day 7.

Maturation pattern in suspension cultures

Figure 4 shows the maturation pattern of normal and CML light density cells at the starting total cell concentrations as given in Figure 2 upper panel (8.1 and $4.7 \times 10^5/\text{ml}$, respectively). With normal cells, there was a steady decrease in neutrophil precursor cells over the 12-day culture period, and the production of mature neutrophils was minimal and did not increase more than 2-fold in the presence of HP-CM. Instead, these cultures produced mainly macrophages

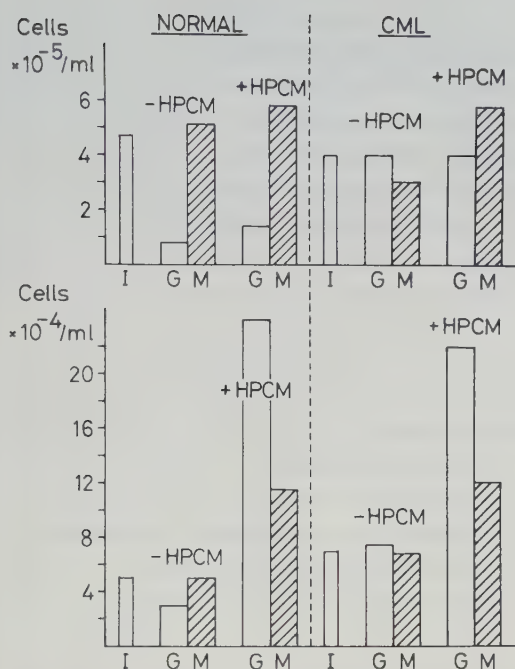


Figure 7. This figure summarizes the findings on neutrophil and macrophage production from normal and CML light density marrow cells cultured at high (upper part) or low (lower part) initial cell concentration. I, initial number of myeloblasts + promyelocytes + myelocytes per ml; G, granulocytes; M, macrophages. The data given represent the maximum levels during the culture period and are compiled from Figures 4 and 5.

which outnumbered the neutrophils by a factor of 10 in the absence of HP-CM; addition of HP-CM resulted in an increased macrophage production. With CML light density cells, the number of neutrophil precursor cells remained at or slightly above the initial value over the first 4–5 d of culture before decreasing. The production of mature neutrophils in CML showed an almost 10-fold increase over the starting value. With HP-CM, the net production was about the same, but maximum was

already reached on days 8–9 suggesting a more rapid maturation process in the presence of HP-CM. In unstimulated cultures of CML cells, the number of macrophages reached approximately the same level as neutrophils, but when HP-CM was added, macrophage production was doubled and outnumbered the neutrophils.

In contrast to these findings, the maturation was different when the initial cell concentration was reduced to $0.8\text{--}1.0 \times 10^5/\text{ml}$ as shown in Figure 5. In the presence of HP-CM, normal neutrophil precursor cells maintained their initial numbers for about 7–8 d, whereas without HP-CM, they steadily decreased in number. The most striking difference was, however, in the production of mature neutrophils: HP-CM generated a more than 10-fold increase over control values at the end of the 12-day culture period. In unstimulated cultures of normal cells macrophages were slightly more numerous than neutrophils, but in the presence of HP-CM, neutrophils were almost twice as numerous as macrophages although macrophage production was significantly increased. In CML, HP-CM also had the effect of maintaining the initial numbers of immature neutrophils over the entire culture period, whereas the control incubations showed a sharp decrease in cell numbers after day 7 of incubation. The production of mature neutrophils was significant even in the absence of HP-CM, which is in contrast to the results from normal cells. On the other hand, the production of macrophages was not significantly different from that of normal cells.

In 2 cases, T-lymphocytes were removed by E-rosetting from normal marrow low density cells before culture. T-cell depletion did not alter either total cell production or maturation pattern (data not shown), suggesting that the higher lymphocyte content

in normal cell cultures was not responsible for the differences noted between normal and CML cultures.

Ion-exchange chromatography and gel chromatography of HP-CM

In order to study whether different activities in HP-CM could stimulate the proliferation and differentiation of cells in agar and liquid culture, respectively, HP-CM was chromatographed on DEAE-Sepharose and Sephadex G-75. The maximum production of neutrophils and macrophages occurred with the fractions of HP-CM where the greatest colony-stimulating activity was found, both for fractions from the DEAE-chromatogram and from the G-75-obtained fractions. CML and normal cells gave similar results (data not shown).

Normal and CML cells at 1.065–1.077 g/ml

As shown in Figure 1, the cells recovered from the density range 1.065–1.077 g/ml are mainly composed of late granulopoietic cells of which most have already lost their proliferation capacity. Total cell production reached values in the range of 1.5–2 times the initial numbers of cells for both normal and CML. The maturation pattern is shown in Figure 6. It is noteworthy that there is no difference in the kinetics for production of mature cells when high ($6.8\text{--}7.0 \times 10^5$ cells/ml) and low ($0.6\text{--}1.0 \times 10^5$ cells/ml) starting cell concentrations were analyzed. This is in contrast to the behaviour of light density cells (<1.065 g/ml) which showed a clear dependence on cell concentration (Figures 4, 5). Another feature different from the low density cells was the low macrophage production (Figure 6), which in no case approached the level of neutrophil production. The addition of HP-CM affected normal cells slightly more than CML

cells but augmented cell production to a lesser extent than that observed with light density cells. HP-CM also seemed to accelerate the maturation of normal cells, whereas CML cells were unaffected in this respect. Macrophage production was only minimally stimulated by HP-CM.

DISCUSSION

In this work we have shown cell concentration dependent differences of the proliferation and maturation patterns between normal and CML light density marrow cells highly enriched for myeloblasts and promyelocytes. Furthermore, differences in the requirement for exogenous CSA were noted.

Our data revealed: (i) that 'unstimulated' CML cells produced more neutrophils than normal cells; (ii) that both CML and normal cells produced neutrophils when the initial cell concentration was low (10^5 /ml) and exogenous CSA added; (iii) that normal cells incubated at a 'high' initial concentration ($5\text{--}8 \times 10^5$ /ml) produced mostly macrophages while CML cells produced both granulocytes and macrophages. Thus, unstimulated CML progenitor cells clearly produced more neutrophils than the corresponding normal cells both at 'low' and 'high' cell concentrations. This difference could be explained by a higher endogenous production of growth factors in CML cell samples or an increased sensitivity to such factors in CML. However, it is also possible that the slightly higher content of myelocytes in the CML cultures could account to some extent for the greater granulocyte production. Macrophage production in unstimulated cultures was similar for normal and CML progenitor cells. These findings are summarized in Figure 7.

Results from ion exchange chromatography and gel chromatography indicated that CSA was the factor responsible for the stimulation of cell production in cultures with a 'low' initial cell concentration. Thus, our findings emphasize that normal myeloid low density cells are dependent on CSA for effective production of neutrophils. CML cells, on the other hand, are less dependent on an exogenous source of such factor(s) for neutrophil production. The cell concentration dependent inhibition of HP-CM-induced granulopoiesis, seen both for normal and CML cells, may depend on (a) inhibitory substances, produced by the cultured cells, (b) decreased availability of receptors for CSA due to crowding of cells or (c) lack of nutrients. Normal and CML precursor cells may be differently sensitive to such cell concentration dependent inhibition of granulopoiesis. In previous studies of epithelial cells in liquid culture, cell concentration dependent growth inhibition was shown to be due, at least in part, to inhibitory substances (11).

The granulopoietic stem cell (CFU-GM) pool is expanded in CML (1, 2) and the CML suspension cultures contained much more CFU-GM than normal cultures. Moreover, normal cell cultures did show some renewal of CFU-GM, whereas CML cultures were steadily depleted of CFU-GM throughout the culture period. Such depletion of CML stem cells may have been due to differentiation of CFU-GM, later during the cultures observed as granulocyte production. Accumulation of normal stem cells, on the other hand, is consistent with inhibition of cell differentiation and thus lack of granulocyte formation. However, granulocyte production was similar for CML and normal cells in stimulated cultures at a 'low' initial cell concentration,

indicating that under such conditions, the mature cells may be preferentially derived from the dominating population of myeloblasts, promyelocytes and myelocytes.

Previous work with normal marrow cells in liquid cultures has shown the growth to be independent of an exogenous source of CSA (12). However, in the latter work, the cells were not separated before culture and the results are therefore not directly comparable with our findings. Using the same liquid culture system with CML cells (13) showed that unseparated CML cells proliferated and produced mature cells even at an initial concentration of 1×10^6 cells/ml, whereas normal marrow cells steadily decreased in numbers over the entire culture period and showed primarily macrophage production. These findings are in accordance with our results. The predominant macrophage production of normal marrow cells that we have observed previously using *in vivo* diffusion chambers (7) at an initial cell concentration of 1×10^6 /ml may be related to a 'high' initial cell concentration incompatible with neutrophil production. However, CML cells produced mostly neutrophils under such conditions.

ACKNOWLEDGEMENTS

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Myeloid Differentiation in Liquid Cultures of Cells from Patients with Chronic Myeloid Leukemia: Effects of Retinoic Acid and Indomethacin

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Abstract. To study the effects of retinoic acid (RA) and indomethacin on myeloid differentiation, normal and chronic myeloid leukemic (CML) bone marrow cells lighter than 1.065 g/ml were incubated in liquid cultures and grown in agar. Retinoic acid (RA) (10^{-8} M and 10^{-6} M) increased the number of clusters (3–40 cells) formed in agar and also increased the neutrophil production in liquid cultures. Indomethacin (10^{-6} M) did not change the cell growth in agar. In liquid culture, however, more macrophages and fewer neutrophils were produced in the presence of indomethacin. The results suggest that RA enhances neutrophil production by stimulating the proliferation and maturation of a cluster-forming cell within the myeloblast–promyelocyte compartment. The stimulatory effect of indomethacin on macrophage production is probably secondary to its inhibition of prostaglandin synthesis, which inhibits the proliferation of monocyte precursor cells. No major differences were noted between CML and normal cells in their responses to RA or indomethacin.

Key words: Chronic myeloid leukemia — Indomethacin — Myeloid differentiation — Retinoic acid

Chronic myeloid leukemia (CML) is a disease of clonal origin arising in a pluripotent stem cell [1]. The most prominent feature of the disease is an expansion of the granulopoietic stem cell compartment leading to neutrophilic hyperplasia [2, 3], but also the erythroid and megakaryocytic stem cells are increased in number [4–6].

During the early stages of the disease, maturation of neutrophils shows only minor abnormalities, which, however, become progressively worse during the chronic phase [7], presumably as an expression of clonal evolution characteristic of CML [8–10]. When cultured in agar, CML CFU-GMs (colony-forming unit–granulocyte, macrophage) show normal maturation except during blastic crisis and sometimes shortly preceding the transformation [11–12].

Isolated immature myeloid cells from normal persons and from patients with CML show different maturation patterns when cultured in liquid cultures: CML cells produce neutrophils as the predominant cell, whereas normal cells produce predominantly macrophages [13]. The proliferation of granulopoietic stem cells is under the control of several feedback mechanisms, one of which is supposedly prostaglandins of the E series produced by the monocyte–macrophage system within the hematopoietic tissues [14]. Prostaglandin E (PGE) inhibits the differentiation of CFU-GMs along the monocytic pathway *in vitro*, but in CML CFU-GMs are less sensitive to this inhibition than normal stem cells [15–

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16]. Theoretically, decreased sensitivity for PGE would lead to an increased production of monocytes; this does not seem to be the case, however, probably because of an altered commitment of the bipotent granulopoietic stem cells or additional regulatory mechanisms.

Recently, RA has been shown to induce terminal differentiation in human leukemic cell lines, e.g., the promyelocytic cell line HL-60 [17] and the histiocytic monoblastoid cell line U937 [18]. It has also been reported that RA enhances colony formation in agar of normal marrow cells [19].

For the present work we studied the effects of RA and inhibition of prostaglandin synthesis by indomethacin on (a) the proliferation and differentiation pattern of CML and normal immature myeloid cells in liquid cultures, (b) colony formation and maturation pattern in agar cultures, and (c) commitment of CFU-GMs during the early stage of liquid culture.

Materials and methods

Collection of bone marrow. Bone marrow cells from healthy volunteers and patients with CML in the chronic phase were suspended in McCoy medium containing heparin (100 IU/ml) and Varidase (75 U/ml; Lederle Laboratories, Pearl River, NY) and mixed with an equal volume of 2% dextran (T500, Pharmacia Fine Chemicals, Uppsala, Sweden), in 0.15 M NaCl to let the red cells sediment for 30–45 min at room temperature. The leukocyte-rich supernatant was collected and centrifuged at 500 g for 5 min; the cells were then resuspended in McCoy medium with 1% fetal calf serum (FCS).

Cell separation. A modification of a previously described method [20] for separation in Percoll gradients was used; 10 ml Percoll with a density of 1.065 g/ml in a 50-ml centrifuge tube (Falcon Plastics, Los Angeles, CA) was carefully overlaid with 10 ml cell suspension with a maximal cell concentration of 2×10^7 cells/ml. Centrifugation was done in a swing-out rotor at 700 g for 20 min. The cells from the interphase, highly enriched with immature myeloid cells, were collected and washed twice in McCoy medium with 1% FCS.

Liquid cultures. Isolated low-density cells (1.065 g/ml) were incubated in 1.5 ml aliquots in 12×75 mm plastic tubes (Falcon Plastics) in McCoy medium with 15% FCS with or without 10^{-6} or 10^{-8} M of all-transretinoic acid (Sigma Chemicals, St. Louis, MO) or 10^{-6} M of indomethacin (Sigma Chemicals, St. Louis, MO) at a cell concentration of $5.9 \pm 0.4 \times 10^5$ cells/ml (CML) and $6.5 \pm$

0.5×10^5 cells/ml (normal). The cells were incubated in 5% CO₂ in a fully humidified atmosphere at 37°C. Cells were harvested on days 0, 2, 4, 8, 11, and 14, counted in a hemocytometer, and used for preparation of cytocentrifuge smears and, on days 0 and 2, for CFU-GM colony assay in agar. Smears were stained with May-Grünwald-Giemsa, and 400 cells were counted for differential morphology. On days 0 and 4, cells were stained with α -naphthyl-butyrate esterase (NBE) for detection of monocytes [21].

³H-thymidine labeling index. In four patients cells were collected from the liquid cultures on days 1, 2, 3, and 4 and incubated with 34 μ Ci/ml of ³H-thymidine (15 Ci/mmol; Amersham, England) for 45 min, washed in McCoy medium and used for cytocentrifuge smears, which were subsequently covered with emulsion K2 (Ilford Ltd., Ilford, England). The autoradiographs were exposed for one week at 4°C and stained with May-Grünwald-Giemsa. Cells with five grains or more were considered as being labeled; 400 cells were counted.

Assay of CFU-GM colony formation in agar. On days 0 and 2 of liquid culture a 0.2-ml aliquot of the cells was cultured in 0.3% agar (Difco Bacto-agar) in 1 ml McCoy medium with 15% FCS and 15% human placenta conditioned medium (HP-CM) [22] on top of 1 ml 0.5% agar in 35-mm Petri dishes (Falcon Plastics, Los Angeles, CA). Four replicates were made, and incubation was done as previously described for liquid cultures. Clusters (3–40 cells) and colonies (more than 40 cells) were counted in an inverted microscope on day 10 of incubation.

Colony morphology. The agar gels were fixed in 5% glutaraldehyde for at least 10 min and transferred to 35×76 mm microscope slides. They were covered with filter paper and several layers of tissue paper; the water soaked up under light pressure for 5–10 min. The gels were cautiously uncovered, dried under a hair drier and stained for 30 min with 0.1% Luxol fast blue MBS (T.T. Gurr, High Wycombe, Bucks, England) in 70% ethanol saturated with urea. The cells were then rinsed in water, and counter-stained with Harris hematoxylin for 5 min, air dried, and mounted with cover slips.

Statistical analysis. All data are given as mean values with SE. Student's *t*-test was used for comparison of results obtained in the presence of RA or indomethacin and the control cultures. Results are indicated in the Figures.

Results

Composition of isolated cells

As has been shown previously, virtually all colony-forming cells are recovered among the low-density (< 1.065 g/ml) cells. As expected [2, 3] the concentration of CFU-GM was increased in

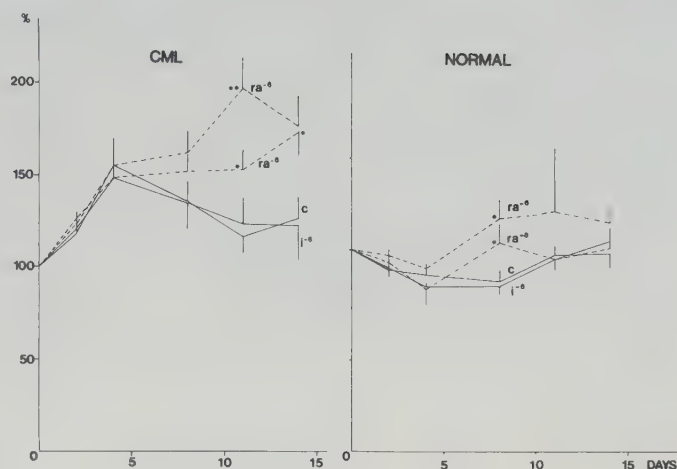


Fig. 1. Total cell counts in the liquid cultures: 100% = 5.86×10^5 cells/ml (CML) and 6.48×10^5 cells/ml (normal); c, control; ra^{-6} , culture with 10^{-6} M RA; ra^{-8} , 10^{-8} M RA; i^{-6} , 10^{-6} M indomethacin. Marrow cells from seven patients with CML and four healthy individuals were studied. Bars show SEM. Significant differences compared with the control incubations are indicated: * $P < 0.05$, ** $P < 0.01$.

CML compared with normal cells. Early granulopoietic precursor cells, i.e. myeloblasts and promyelocytes, were highly concentrated in the low-density fraction and together made up $54 \pm 6\%$ (CML) and $32 \pm 4\%$ (normal), respectively. The main difference between normal and CML cell fractions was the content of lymphocytes, which was $51 \pm 10\%$ (normal) and $7 \pm 2\%$ (CML), respectively.

Cell production in liquid cultures

The total cell counts in the liquid cultures are shown in Figure 1. The CML cells increased to about 150% of the initial values on day 4 and then slowly decreased. However, with RA added, the cell counts continued to increase slowly after day 4 ($P < 0.01$) with 10^{-6} M RA at day 10 as compared with control cells.

Normal cell quantities did not change significantly during the culture period, but the addition of 10^{-6} M RA resulted in slightly higher cell counts ($P < 0.05$ at day 8). Indomethacin did not alter the results in either normal or CML cell cultures.

Differentiation of CML cells

Differentiation of CML cells is shown in Figure 2. The number of myeloblasts, promyelocytes, and myelocytes increased initially until day 4

and then decreased. In the presence of RA, the decrease was slower, leading to a greater number of immature myeloid cells than in the controls at the end of the culture period. Indomethacin had no effect on the numbers of myeloblasts-myelocytes.

After day 2, metamyelocytes and mature neutrophils (PMNs) increased about threefold. In the presence of RA, this increase was significantly higher, whereas cultures with indomethacin showed slightly reduced numbers of mature granulocytes. In contrast, the production of monocytes and macrophages was stimulated in cultures containing indomethacin and reduced in cultures containing RA.

Differentiation of normal cells

The number of normal myeloblasts, promyelocytes and myelocytes decreased throughout the culture period (Fig. 2). The number of immature myeloid cells increased with RA (10^{-6} M), whereas indomethacin (10^{-6} M) did not change their number.

Few mature granulocytes were formed from normal cells in liquid cultures. Indomethacin reduced and RA increased the yield of mature cells. Macrophage production was slightly greater in normal than in CML cell cultures. Also, in cultures of normal cells indomethacin stimulated and RA inhibited the formation of macro-

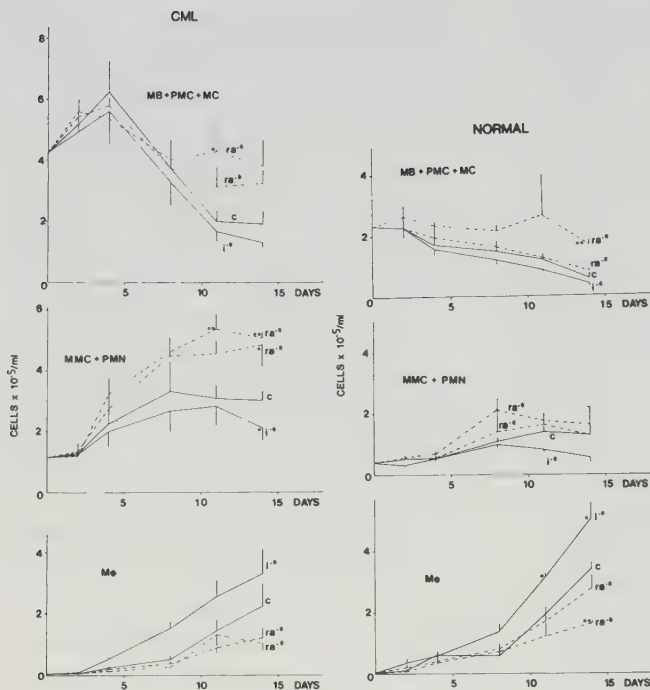


Fig. 2. Differential counts of cells in liquid cultures, absolute numbers: MB, myeloblast; PMC, promyelocyte; MC, myelocyte; MMC, metamyelocyte; PMN, mature granulocyte; Mo, monocyte/macrophage; $n = 7$ (CML) and 4 (normal), respectively. For explanation of the symbols, see Figure 1. Bars show SEM. Significant differences compared with the control incubations are indicated: * $P < 0.05$, ** $P < 0.01$.

phages. The monocyte/macrophage morphology was confirmed with α -naphthyl-butyrate esterase staining for early detection of monocytic cells.

Labeling index of CML cells in liquid cultures

On days 0, 1, 2, 3, and 4 of liquid culture of CML cells with or without RA, autoradiographs were prepared for estimation of labeling index. Labeling index of myeloblasts–myelocytes showed a mean value of $27 \pm 3\%$ on day 0, which increased to $37 \pm 4\%$ (control) and $41 \pm 3\%$ (RA) on day 1 and then slowly decreased during the next three days of culture to $22 \pm 4\%$ (control) and $14 \pm 3\%$ (RA); RA did not significantly alter the LI.

CFU-GM in liquid cultures

The number of CFU-GM was estimated on day 0 and on day 2 of liquid culture to evaluate the early effects of RA and indomethacin on colony-

forming cells (Fig. 3). As expected, the concentration of colony- and cluster-forming cells was higher in CML than in normal cells. The number of colony-forming cells was not changed by RA or indomethacin, whereas the number of cluster-forming cells was increased in the presence of 10^{-6} M RA in both CML and normal cells. The number of CFU-GMs in CML control cultures decreased from day 0 to day 2 in contrast to that of normal CFU-GMs, which increased slightly over the same period.

Colony morphology was assessed after staining of the agar gels with Luxol fast blue (eosinophils show green color) and Harris hematoxylin (Fig. 4). Pure neutrophil colonies by far outnumbered other colony types, such as macrophage colonies, mixed macrophage–neutrophil, and eosinophil colonies; RA, 10^{-6} M, seemed to inhibit the formation of eosinophil colonies, whereas indomethacin (10^{-6} M) stimulated eosinophil colony formation in some cultures on day 0. These effects were more pronounced in CML cell cultures than in normal cell cultures.

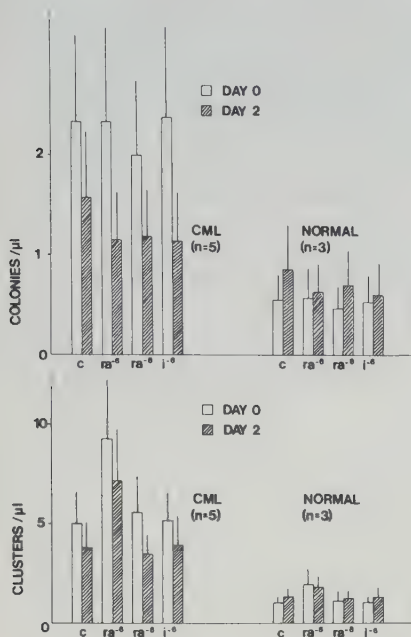


Fig. 3. Growth in agar of colonies and clusters initially (day 0) and after two days (day 2) in liquid culture containing RA or indomethacin. Symbols as in Figure 1. Bars show SEM.

RA in agar cultures

To further elucidate the effects of RA on colony- and cluster-forming cells, 10^{-6} M RA was added to agar cultures together with varying concentrations HP-CM (Fig. 5). The RA seemed to inhibit colony formation of CML cells at low and intermediate HP-CM concentrations, whereas this inhibition was overcome at the highest HP-CM concentrations tested (see also Fig. 4). Clusters were, however, increased at all concentrations of HP-CM (Fig. 5). Colony formation of normal cells was not significantly altered by RA at the various HP-CM concentrations tested (Fig. 5), but clusters were generally increased in number.

When different concentrations of RA were added to agar cultures together with an optimal concentration of HP-CM (Fig. 6), a slight reduction of CML colony numbers was noted with increasing concentrations of RA. Normal colony numbers were not affected by RA under these

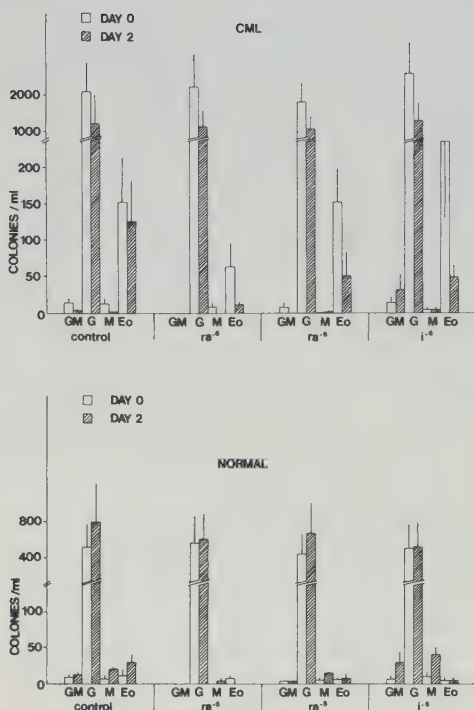


Fig. 4. Differential counts of colonies (after staining of the agar gels) initially and after two days of incubation in liquid culture with RA or indomethacin: GM, mixed granulocyte-macrophage colony; G, granulocyte colony; M, macrophage colony; Eo, eosinophil colony; other symbols as in Figure 1; $n = 5$ (CML) and 3 (normal). Bars show SEM.

circumstances. On the other hand, CML clusters increased in number with increasing concentrations of RA (Fig. 6), while the corresponding increase of normal clusters was less prominent.

For comparison, normal marrow cells were isolated from Isopaque-Ficoll gradients, and the cells lighter than 1.077 g/ml were cultured in agar at increasing concentrations of HP-CM with or without 10^{-6} M RA. With these cells both colony and cluster formation were stimulated by RA (data not shown) in accordance with previously reported findings [19].

Discussion

Bone marrow cells lighter than 1.065 g/ml isolated in Percoll gradients contain virtually all

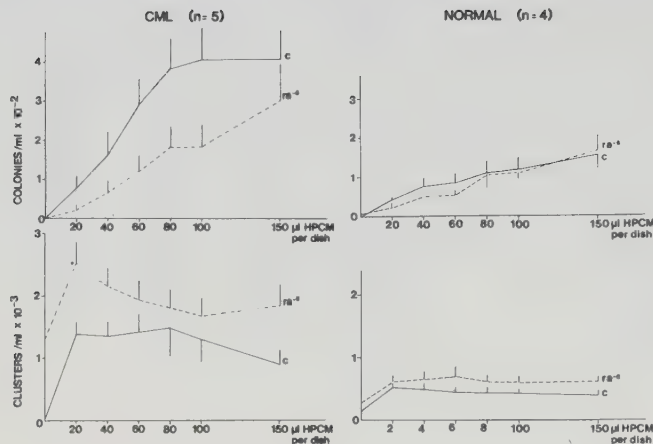


Fig. 5. Growth in agar of colonies and clusters at various concentrations of HP-CM with or without RA, $10^{-6}M$. Symbols as in Figure 1.

colony- and cluster-forming cells and are highly enriched for myeloblasts and promyelocytes [20]. We have shown previously [13, 23] that light density cells from patients with CML, depending on cell concentration, show a different maturation pattern in vitro than the corresponding normal cells; CML cells produced predominantly neutrophils, whereas normal cells produced predominantly macrophages in diffusion chambers [23] and in liquid cultures at cell concentrations above $5 \times 10^5/\text{ml}$ [13].

Retinoic acid induces differentiation of the leukemic promyelocytic cell line HL-60 [17] to mature neutrophils and of the monoblastoid cell line U937 to monocytelike cells [18]. It also induces terminal differentiation of cells from patients with promyelocytic leukemia, whereas cells from other forms of acute myeloid leukemia seem to be unaffected [24]. In the present study we found that RA also increases the production of mature neutrophils in liquid cultures of both normal and CML cells, while macrophage production was inhibited.

Two interpretations of RA's dual effect on neutrophil and macrophage products can be proposed: (a) RA affects the commitment of the bipotent granulopoietic stem cell CFU-GMs, shifting the commitment toward neutrophil production at the expense of macrophage production, and (b) RA stimulates the proliferation of precursor cells already committed for granulopoiesis and inhibits the proliferation of monocytic precursor cells. In an attempt to elucidate these questions we have studied the effects of RA on colony- and cluster-forming cells in agar cultures.

The finding at suboptimal doses of CSA of a RA-mediated reduction in the number of colonies formed in CML and to a lesser extent in normal marrow cells suggests a reduced sensitivity, particularly of CML cells, to CSA in the presence of RA. The maturation pattern of the colonies was not changed by RA, except that eosinophil colonies were reduced by approximately 50% at $10^{-6}M$ (and even more after two days of preculture). This lack of effect on neutrophil and macrophage colony distribution was evident both from experiments in which RA was added directly to the agar cultures and from experiments where the cells were preincubated in liquid culture for two days before seeding in agar.

Thus, the increased production of neutrophils in suspension cultures with RA cannot be explained by hypothesizing an altered commitment of the granulopoietic stem cells toward neutrophil production.

In contrast to the inhibition of colony-forming cells, the number of clusters was increased by RA in a dose-dependent fashion. This stimulation—more prominent for CML cells—was a net increase and was not due to a reduction in the size of the colonies that would make them ap-

pear as clusters, since the total number of colonies and clusters increased in the presence of RA. The augmented cluster formation was almost exclusively due to an increased number of neutrophilic clusters, whereas macrophage clusters were almost unaffected and eosinophil clusters slightly reduced (data not shown). These results indicate that the stimulatory effect of RA in agar cultures acts on a cluster-forming cell already committed for neutrophil production. Stimulation was independent of the concentration of exogenous CSA and was observed even in the absence of HP-CM. Both HP-CM and RA thus showed additive effects on the stimulation of cluster formation.

Colony- and cluster-forming cells do not separate from each other in Percoll gradients, but both show the same distribution as the myeloblasts and a subpopulation of light-density promyelocytes [20]. We suggest that it is the same cell that gives rise to an increased number of clusters in agar and an augmented neutrophil production in liquid cultures in the presence of RA. The most likely candidate is a cell within the myeloblast–promyelocyte compartments committed for neutrophil production, although results of the present experiments do not prove this. The RA-mediated inhibition of macrophage production may be a secondary phenomenon rather than a direct effect on monocyte precursor cells.

A stimulatory effect of RA on the proliferation of myeloid precursor cells should theoretically result in an increased fraction of these cells in S phase. A determination of thymidine labeling index during liquid culture demonstrated no major differences between control cells and cells exposed to RA. If, however, the total number of cells was taken into account, RA produced an increased number of myeloid precursor cells in S phase during the initial phase of liquid culture. Another explanation for the seemingly minor effects on the fraction of cells in S phase could be a shortened generation time, which would increase the cell production without affecting the fraction of cells in S phase at any one moment.

Douer and Koeffler [19], in contrast to our observations, reported recently that RA stimulates colony formation of normal bone marrow

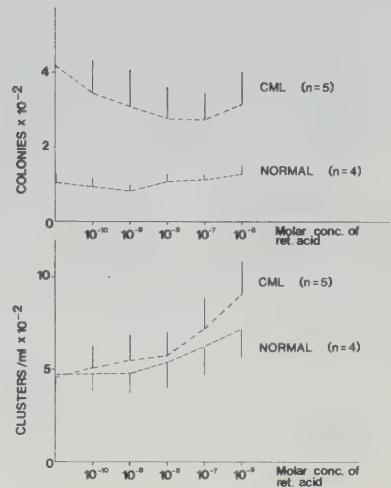


Fig. 6. Growth in agar of colonies and clusters at various concentrations of RA with optimal HP-CM concentration, 15%.

cells. However, they used cells separated on Isopaque–Ficoll lighter than 1.077 g/ml, and we have confirmed their observations using such cells. Therefore, the discrepancy between cells lighter than 1.065 g/ml and 1.077 g/ml is probably not due to an effect on colony-forming cells per se. It is more likely due to interactions of RA with accessory cells in the cell population lighter than 1.077 g/ml—interactions not occurring in the cells lighter than 1.065 g/ml. However, minor differences in the composition of accessory cells in CML vs normal cell populations lighter than 1.065 g/ml cannot be excluded.

Indomethacin was used at a concentration that effectively inhibits prostaglandin synthesis. According to current models of the regulation of granulopoiesis, PGE inhibits the formation of monocytes/macrophages in vitro by decreasing the sensitivity for CSA of stem cells committed for monocyte production [25]. Theoretically, indomethacin would result in an increased monocyte production, which we observed in liquid cultures of both normal and CML cells. The number of macrophage colonies in agar, however, was not affected by indomethacin; an increased number of eosinophil colonies was ob-

served in CML but not normal cells. Indomethacin had only minor inhibitory effects on total cell production and production of mature neutrophils in liquid culture and did not inhibit neutrophil colony or cluster formation in agar.

This study has shown that the proliferation and differentiation of highly enriched populations of myeloid precursor cells can be modulated by RA and indomethacin with partially opposing effects and that CML cells and normal cells respond in a similar fashion. It is suggested that RA acts on a cell within the myeloblast-promyelocyte compartment with cluster-forming capacity. The effects of indomethacin, on the other hand, are probably mediated over the PGE synthesis in the monocytes with secondary effects on the monocytic precursor cells.

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DENSITY DISTRIBUTION OF CHRONIC MYELOID LEUKEMIA AND NORMAL COLONY-FORMING CELLS IN DIFFUSION CHAMBERS (CFU-D) AND AGAR (CFU-GM)

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Running head: Density of CFU-D in CML

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ABSTRACT

The density distribution in Percoll gradients of clonogenic cells forming colonies in diffusion chambers (CFU-D) or in agar culture (CFU-GM) was studied in chronic myeloid leukemia (CML). The density distribution of CFU-GM in CML was homogeneous with peaks within 1.058-1.061 g/ml, which is slightly lower than normal. CFU-D on the other hand, showed heterogeneous distributions both in CML and in normal controls. Two separable populations of CFU-D were recognized, one with the same or lower density than CFU-GM that formed almost exclusively neutrophilic colonies in diffusion chambers, and a second population concentrating in the density range 1.068-1.075 g/ml which primarily formed macrophage colonies. The second population contained colony-forming cells derived from both Fc-receptor positive and Fc-receptor negative precursor cells suggesting that at least some colonies in diffusion chambers arise from Fc-receptor positive granulopoietic cells of intermediate maturity and/or monocytes. The concentration of CFU-D in peripheral blood was increased 40-100 fold in two patients currently off treatment who had increased WBC counts.

Key words: CFU-D, CFU-GM, density separation, chronic myeloid leukemia

INTRODUCTION

The leukocytosis of chronic myeloid leukemia (CML) is due, at least in part, to an increased production of stem cells. Thus increased numbers of stem cells for granulopoiesis (1, 2), erythropoiesis (3, 4) and megakaryocytopoiesis (5) have been demonstrated as well as a rise of pluripotential stem cells (6).

In a previous study of growth and differentiation in diffusion chambers of CML and normal progenitor cells, CML cells were found to produce more neutrophils than normal cells, while macrophage formation was identical for CML and normal progenitors (7). Similar results were also obtained by suspension cultures in vitro (8). In an attempt to further define differences between normal and CML cells the work has now been extended to studies of colony formation in diffusion chambers.

The CFU-D produces colonies in fibrin clots of diffusion chambers, that are placed intraperitoneally in bone marrow depressed mice (9, 10, 11). Previous data have indicated non-identity between the CFU-GM and the CFU-D based on findings of different kinetic properties (12) and sedimentation velocity as well as differences in the fraction of cells in active DNA synthesis (13).

In the present investigation the cell densities of CFU-GM and CFU-D of normal and CML origin are compared and the concentrations of CFU-D in peripheral blood from CML patients and normal controls are estimated. The results show an expansion of the investigated stem cell compartments in CML and demonstrate heterogeneous density distributions of CFU-D in CML and normals.

MATERIAL AND METHODS

Patients

Bone marrow or blood was obtained from seven patients with Ph¹-chromosome positive chronic myeloid leukemia, who all were in the chronic phase of the disease. They had been treated with busulfan or hydroxurea for varying time periods. Bone marrow and blood was also obtained from healthy volunteers.

Collection of cells

Bone marrow was aspirated by sternal puncture and collected in a sterile tube containing 3 ml of McCoy's medium with heparin (100 IU) and varidase (75IU; Lederle Laboratories, Pearl River, NY). Marrow particles were disrupted by repeated suction through a fine needle, and the cells were mixed with an equal volume of 2 % Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.15 M sodium chloride and allowed to stand for 45-60 min at room temperature.

The leukocyte-rich plasma was collected and centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in 3-5 ml of McCoy's medium supplemented with 1% fetal bovine serum (FBS) (Flow Laboratories). Peripheral blood was collected in heparinized tubes (Vacutainer, Becton-Dickinson) and sedimented with dextran before separation as described.

Percoll separation

Percoll separation was performed as previously described (14). Linear density gradients were made from mixtures of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and Hank's BSS. The gradient was formed by use of a peristaltic pump (Varioperpex 2120, LKB-Beckman Instruments, Bromma, Sweden) in 17x100 mm polypropylene tubes (Falcon Plastics). Two ml of marrow suspension (3×10^7 cells per ml) was layered on top of the gradient and the tubes were spun at $1600 \times g$ for 20 min in an angle rotor. After centrifugation, 1 ml fractions were collected from the bottom of the tubes using the peristaltic pump. The cells were washed twice and resuspended in McCoy's medium with 1% FBS and counted in a hemocytometer.

In four experiments, pooled fractions of CML marrow cells (density 1.068-1.075 g/ml) were incubated with IgG-coated sheep red blood cells for 60 min at 4°C for detection of Fc-receptor bearing cells (15). After this incubation, the cells were separated in Isopaque-Ficoll (Lymphoprep, Nyegaard & Co, Oslo, Norway) (1.077 g/ml) in order to separate rosette-forming (Fc+) cells from non-rosette-forming (Fc-) cells. A fraction of mixed Fc+ and Fc- cells was preserved for culture.

In other experiments peripheral blood cells were separated in Isopaque-Ficoll (1.077 g/ml) and the low density cells cultured in diffusion chambers for assessment of the concentration of CFU-D in peripheral blood.

Agar culture

10^5 cells from each fraction of the density gradient were cultured in agar as previously described (8) with 10% human placenta conditioned medium (HP-CM) as source of colony stimulating activity (CSA). Colonies of more than 40 cells were counted in an inverted microscope on day 10 of culture.

Diffusion chamber (DC) culture

The DC technique was carried out as previously described (9, 10, 11) with some modification. The Millipore chambers (volume 140 μl) which consist of a plastic ring with a nitrocellulose filter (Millipore, pore size 0.22 μm) glued to each side of it were tested for leaks and sterilized for 2-4 h in ultra-violet light.

The cells were suspended in McCoy's medium with 5% FBS and mixed with an equal volume of 1% fibrinogen (Kabi, Stockholm, Sweden) in 0.15 M sodium chloride. 100 μ l of this suspension containing $5-10 \times 10^4$ cells were injected into each chamber whereafter 10-15 NIH units of thrombin in 25 μ l (Topostasine^R, Roche, Switzerland) was added in order to have an immediate clot formation. The chambers were sealed with a plastic plug, which was melted to the plastic ring, and then kept in culture medium before implantation. Twenty chambers were used for each cell fraction.

Male NMRI mice weighing 25 g were used for implantation. 24 hours before implantation, cyclophosphamide, 275 mg/kg, was injected intraperitoneally to produce marrow hypoplasia. During pentobarbital anaesthesia, 2 chambers were implanted intraperitoneally into each mouse. After one week, the chambers were reimplanted into new cyclophosphamide-pretreated mice, and after another week, the chambers were harvested.

The filters with the attached plasma clots were removed, fixed in 5% glutaraldehyde and stained with Harris' hematoxylin. After drying, the preparation was made transparent in immersion oil (Millipore transparency medium), and the number of colonies with more than 30 cells were counted.

RESULTS

Fig 1 shows the density distribution profile of CFU-GM and CFU-D from four normal marrow samples. The cells which form colonies in agar (CFU-GM) showed similar distributions with peaks at 1.060-1.063 g/ml. The colonies were almost exclusively of pure neutrophil type. The CFU-D's, on the other hand, showed heterogeneous distributions and did not peak at a distinct density. The colonies were of at least three different types with macrophages only in colonies with the highest density, neutrophils only in colonies with low density, or with unidentifiable mononuclear cells. Exact numbers for the different types of colonies were not possible to obtain. Three additional cases (data not shown) showed similar results and in only 2/7 cases did CFU-D peak at the same density as CFU-GM; in three cases CFU-D had higher peak densities than CFU-GM and in two cases CFU-D had lower peak densities than the corresponding CFU-GM.

The density distribution profile of CML-derived CFU-GM and CFU-D is shown in fig. 2. The peak density of the CFU-GM was at 1.058-1.061 g/ml, which is slightly lower than for the corresponding normal cells. CML-derived CFU-D's

also showed a heterogeneous density distribution and in two cases two different populations, one with a low and one with a high density, were distinctly separated from each other.

The nature of the high density peak of CML-derived CFU-D's was further analyzed using a cell fraction obtained at 1.068-1.075 g/ml (table 1). Such cells were separated with regard to Fc receptors and cultured in DC. The Fc+ cells made up $24 \pm 4\%$ (SEM) and the Fc- cells $76 \pm 4\%$ with a recovery of 89%. Plating efficiency was higher for both Fc+ and Fc-cells compared to a mixture of these cells. Size and morphology of the colonies were similar for the two separated cell fractions.

Three normal and four CML blood samples were separated in Isopaque-Ficoll (1.077 g/ml) and cultured in plasma clots in DC for assessment of the number of peripheral blood CFU-D (table 2). Normal blood contained 43.4 ± 5 CFU-D per ml blood, and two patients with CML under continuing treatment and WBC of $18 \times 10^9/l$ and $26 \times 10^9/l$, respectively, had peripheral blood CFU-D in the normal range, but two CML patients currently off treatment and with WBC of $60 \times 10^9/l$ and $112 \times 10^9/l$, respectively, had 40-100-fold increased concentrations of blood CFU-D.

DISCUSSION

In this study the density distribution of CFU-GM and CFU-D from patients with CML have been investigated. The CFU-GM's were recovered in single peaks indicative of a relative homogeneity of these cells. As reported earlier (14) CFU-GM had a lower buoyant density in CML than normal marrow CFU-GM. The CFU-D's, however, were heterogeneous with respect to density and distributed over a wide density range often without distinct peaks. This may be related to the different morphology found for colonies of low and high density; although incomplete, our data suggest that macrophage colonies are derived from cells with a higher density than those CFU-D's producing granulocytic colonies. The heterogeneity of CFU-D is further illustrated by the experiments where cells with a density between 1.068 and 1.075 g/ml were separated into FcR-positive and FcR-negative populations. Both populations gave rise to colonies of similar size and morphology in diffusion chambers. Marrow cells within this density range are predominately promyelocytes, myelocytes and monocytes (14), which possibly may give rise to colonies in the diffusion chambers. Promyelocytes do not have any FcR but 5 to 10 % of myelocytes have (16) and a majori-

ty of monocytes have FcR (17). The fact that FcR+ and FcR- cell populations had higher plating efficiencies than a mixture of such cells suggest that interactions between these cell populations can modify the growth of CFU-D.

Heterogeneity of the CFU-GM has been documented previously. Supernatants from human fibroblast cell lines stimulate preferentially large cells that form neutrophilic colonies, while medium from phyto hemagglutinin-stimulated peripheral blood leukocytes mainly stimulate small cells that mostly give rise to macrophage colonies (18). The density distribution of normal CFU-GM is affected by the activity of the feeder layer used for colony stimulation. Highly active feeder layers stimulate low density colony forming cells, which are not detected when feeder layers with low CSA are used (19). In our study, the CFU-GM had a relatively homogeneous density distribution, and HP-CM was used at an optimal concentration. Different peak densities of CFU-GM may reflect individual differences in the sensitivity to CSA or in the composition of the population of CFU-GM by the time of the study.

There is evidence that CFU-D is an intermediate stage between CFU-S and CFU-GM (12, 13, 20, 21) and a possible parent-progeny relationship between CFU-D and CFU-GM has been demonstrated (22). In velocity sedimentation at 1 g CFU-D is a smaller cell than CFU-GM (13) with a relatively homogeneous size distribution. However, in these experiments bone marrow cells were first separated in an albumin gradient and only the cells with a density lower than 1.070 g/ml were used for velocity sedimentation. Therefore, a majority of those CFU-D with a density above 1.068 g/ml that we have found in Percoll gradients were not included in the velocity sedimentation experiments, which may have contributed to the seemingly homogeneous size distribution of CFU-D (13).

Our findings indicate non-identity between CFU-D and CFU-GM in agreement with previous studies (12, 13, 20), and emphasize the fact that CFU-D is a heterogeneous population (23, 24). Some of the colonies in diffusion chambers, and especially those derived from cells of medium to high density, arise from cells of intermediate maturity such as promyelocytes and marrow monocytes, and not necessarily from primitive CFU-D's with stem cell properties. It is conceivable that the colonies recovered at densities lower than 1.065 g/ml in Percoll gradients correspond to those CFU-D's isolated by velocity sedimentation (13).

We did not find any significant differences between the density distribution of CFU-D in CML and normals, but in CML there was a markedly increased concentration of CFU-D in peripheral blood with increasing WBC counts. Patients on busulfan treatment and with only moderately increased WBC had normal concentrations of CFU-D in peripheral blood. These findings are in agreement with what has been reported for CFU-GM (1, 2) and BFU-E (3, 4).

We conclude that cells forming colonies in diffusion chambers are heterogeneous with respect to density and surface receptors, but are separable from CFU-GM. Suggestively, it is only those CFU-D's with a density below 1.065 g/ml that represent stem cells, whereas colony forming cells of higher density probably represent neutrophilic or monocytic precursor cells with a limited proliferative capacity. Except for increased concentrations of CFU-D in peripheral blood and bone marrow, CFU-D in CML were not different from normals.

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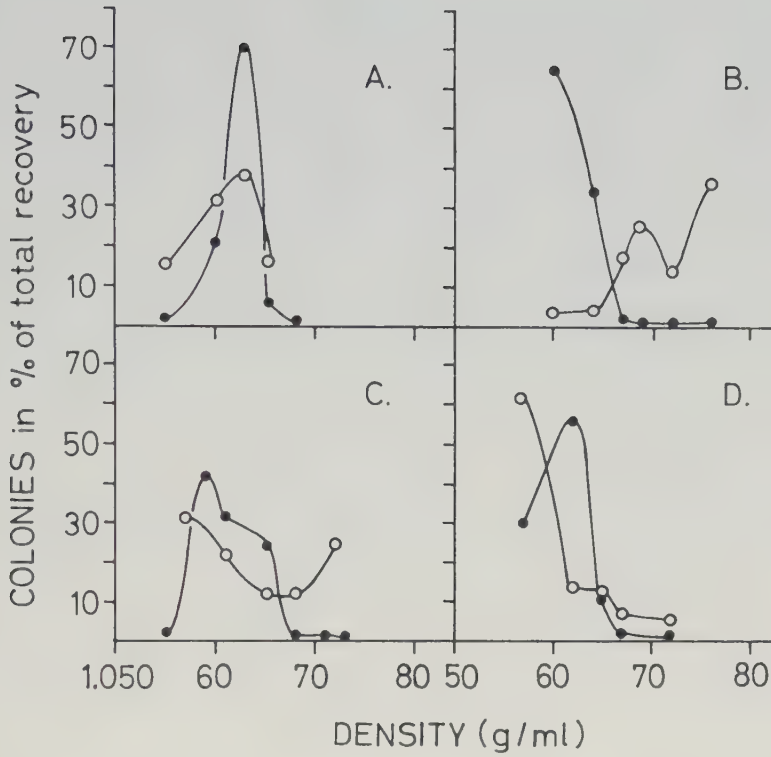


Figure 1 Density distribution of CFU-GM (●—●) and CFU-D (○—○) from four normal marrows. The results are shown as number of colonies in per cent of all colonies recovered within the density range indicated by each curve. The total number of colonies were (CFU-GM/CFU-D): A, $91 \times 10^3/2.5 \times 10^3$; B, $11 \times 10^3/2.6 \times 10^3$; C, $4.3 \times 10^3/3.0 \times 10^3$; D, $11 \times 10^3/1.0 \times 10^3$.

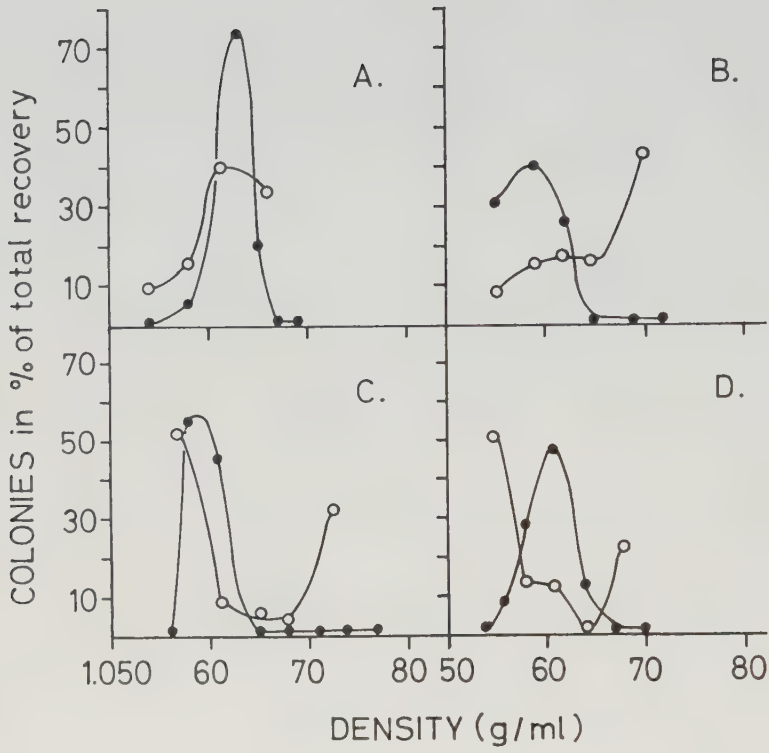


Figure 2 Density distribution of CFU-GM (●—●) and CFU-D (○—○) from four patients with CML. The total number of colonies were (CFU-GM/CFU-D): A, $5.4 \times 10^3/1.6 \times 10^3$; B, $52 \times 10^3/9 \times 10^3$; C, $3.9 \times 10^3/2.9 \times 10^3$; D, $11 \times 10^3/2.1 \times 10^3$.

TABLE 1.

Colony formation in DC from FcR+ and FcR- CML marrow cells.

Patient	Colonies per DC		
	FcR+	FcR-	unseparated
1	20.8	21.5	13.6
2	22.5	12.2	7.0
3	32.7	22.5	24.1
4	31.5	16.0	38.9
Mean $\pm$ SEM	26.9 $\pm$ 2.6	18.1 $\pm$ 2.1	20.9 $\pm$ 6.0

Table legend.

CML marrow cells were pooled from Percoll gradients in the density range 1.068 - 1.075 g/ml and separated into FcR+ and FcR- populations with IgG-coated sheep red blood cells. The volumes were kept constant to make the number of colonies per DC directly comparable for separated and unseparated cells.

TABLE 2.

CFU-D in peripheral blood.

Patient	WBC x 10 ⁹ /l	CFU-D/10 ⁵	CFU-D/ml blood
CML 1	60	10.4	1771
" 2	112	11.5	4381
" 3	18	1.7	37.4
" 4	26	1.4	33.8
NPB 1	5.2	2.8	48.8
" 2	5.4	1.5	31.5
" 3	6.1	1.4	50.0

Table legend.

Peripheral blood from CML patients and normals (NPB) was separated in Lymphoprep and the cells with a density ≤ 1.077 g/ml were cultured in diffusion chambers; the results are shown as CFU-D/10⁵ cells. White blood cell counts (WBC) and the number of cells lighter than 1.077 g/ml were used to calculate the number of CFU-D per ml blood.

DEFECTIVE RECLONING CAPACITY OF GRANULOCYTE-MACROPHAGE COLONY FORMING CELLS IN
CHRONIC MYELOID LEUKEMIA

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ABSTRACT

We investigated the recloning capacity of normal and chronic myeloid leukemia granulocyte-macrophage colony forming cells (CFU-GM) after seven days culture in methylcellulose. Normal CFU-GM expressed self-replication with a mean content of 3.84 ± 1.02 secondary CFU-GM per primary day 7 colony. A frequency distribution of the colony-forming cells within day 7 colonies showed a heterogeneous distribution with the majority of day 7 colonies containing one or zero CFU-GM and a few colonies containing more than 30 CFU-GM. Separation of marrow cells by velocity sedimentation demonstrated that the recloning capacity was primarily expressed by the smallest colony-forming cells. In contrast, marrow or blood cells from 13 patients with Ph¹-chromosome positive CML in the chronic phase produced colonies with defective recloning capacity. Only one patient had a recloning value within the normal range, and the others had a mean value of 0.38 (range 0-1.28) secondary colonies per primary day 7 colony. The defective self-replication was not related to WBC, duration of the disease or treatment, and five newly diagnosed patients with CML showed defective self-replication also before any treatment was given, which suggests that the defect is an inherent property of CML.

INTRODUCTION

Chronic myeloid leukemia, CML, originates in a multipotent stem cell (1-3), but the disease is primarily expressed within the neutrophilic compartment with marked leukocytosis and extramedullar hematopoiesis in the spleen. The number of granulopoietic progenitor cells, CFU-GM, is increased in both blood and marrow and the concentration of CFU-GM in blood correlates to the degree of leukocytosis (4, 5) suggesting that the neutrophilic hyperplasia is caused by an increased cell production by the expanded granulocytic stem cell pool.

Self-renewal capacity is a property of stem cells and can be demonstrated for pluripotent stem cells in mice (6), for a population of blast progenitor cells in acute myeloid leukemia (7), and for multipotent stem cells growing in vitro (8-10). Granulocyte-macrophage progenitor cells are generally considered to have low or absent self-renewal capacity (11).

In a recent study of cell proliferation and differentiation of normal and CML cells in suspension cultures we observed that normal marrow cells regularly produce a net increase in the number of CFU-GM in suspension cultures on day 3-5, whereas CML cells consistently failed to produce such an increase (12). This observation suggested either that normal CFU-GM expressed a restricted self-renewal capacity but CFU-GM in CML did not, or that normal marrow suspension cultures contained pre-CFU-GM capable of producing new CFU-GM. In an attempt to further elucidate this question we have studied the recloning capacity of normal and CML CFU-GM grown in methylcellulose and found that normal colonies contain a varying number of CFU-GM but that colonies in CML only rarely do so.

MATERIALS AND METHODS

Patients

Thirteen patients with Ph¹-chromosome positive CML in the chronic phase were studied. Five of the patients were studied shortly after diagnosis before any treatment was given and the other patients had 7-265 months duration of their disease. The patients are presented in Table 1. Nine healthy volunteers served as control marrow donors; three of them were studied twice with an interval of six, eleven and eleven months respectively.

Cell preparation

Bone marrow was collected in heparinized McCoy's medium and the suspended marrow cells centrifuged through Ficoll-Isopaque (Lymphoprep, Nyegaard & Co, Oslo, Norway) for 20 min at 700 x g. The mononuclear cells recovered from the interphase were washed twice in McCoy's medium with 1% fetal bovine serum (FBS, Flow Laboratories) and then resuspended in Iscove's modified Dulbecco medium (IDMEM, Flow Laboratories). Peripheral blood was collected in heparinized tubes (Vacutainer, Becton-Dickinson), diluted with one volume of sterile 0.15 M NaCl and then applied to Ficoll-Isopaque gradients as described.

Velocity sedimentation

In four experiments (2 normals and 2 CML patients) $40-80 \times 10^6$ marrow cells isolated from Ficoll-Isopaque gradients were separated according to cell size using velocity sedimentation in an albumin gradient of 0.4-2.0% bovine serum albumin in PBS at 4°C for 3 hours (13, 14). Fractions of 30 ml were collected and the cells spun down and resuspended in IDMEM with 1 % FBS before cell counting and culture in methylcellulose.

Assay of CFU-GM and recloning capacity

Isolated mononuclear cells from blood or bone marrow were cultured in methylcellulose as follows: 0.5 or 1×10^5 cells/ml in IDMEM, 0.8% methylcellulose (Methocel, A4M, Dow Chemical Co., Midlands, Mich.), 30% FBS, and 10% human placenta conditioned medium, HP-CM (15), or 10% GCT conditioned medium (16) (Gibco Laboratories, Grand Island, N.Y.). Eight one ml replicates were made in 35 mm petri dishes (Falcon Plastics) and incubated in a fully humidified incubator with a constant flow of 5% CO₂ in air. On day 7 colonies of more than 40 cells were counted in an inverted microscope. An appropriate number of dishes with a known number of colonies were harvested by adding 3 ml of IDMEM with 10% FBS to each dish, the cells were suspended, pooled and washed and replated as described for the first plating procedure. Secondary colonies were counted on day 7 and the recloning capacity calculated according to the following formula: number of secondary colonies / number of replated primary colonies. In addition, in four normal cases single colonies were picked at random from the dishes under the microscope with the tip of a 25 ul glass capillary tube. The colonies were pooled in culture medium and washed once before being cultured the second time; alternatively, single colonies were immediately transferred to one of the wells in a 24-well multidish (diam. 15 mm; A/S Nunc, Roskilde, Denmark). Each well contained 0.3 ml of fresh culture medium inclu-

ding methylcellulose, FBS and HP-CM. The transfer was checked microscopically before the colony cells were dispersed by shaking the plate. The cells were incubated as described and colonies counted after 7 days.

RESULTS

Recloning of normal colony-forming cells

Normal marrow derived day 7 colonies contained cells with recloning capacity. In twelve experiments with nine different normal marrows the mean number of secondary colonies per primary colony ranged from 2.38 to 5.93 with a mean value of 3.84 ± 1.02 (S.D.) (Fig. 1). Three of the normals were studied twice with an interval of six, eleven and eleven months respectively; the recloning values were 2.95 and 2.75 in the first subject, 4.42 and 5.93 in the second and 2.38 and 3.77 in the third. Colonies harvested on day 7 contained 40-100 cells and the resulting secondary colonies were of the same size and morphology, mainly neutrophilic. Control experiments in one of the normals showed that the recloning capacity was about the same on days 7, 8 and 9, but then decreased gradually and was absent on day 13 and 14. In two experiments pooled colonies were recultured at four different concentrations and the number of secondary colonies was linear with the number of seeded cells in both experiments. In three normals secondary day 7 colonies were recultured and the recloning values were 0, 0.14 and 0.34, which indicates that very few of secondary colonies contain colony forming cells.

Since it can be argued that potential colony forming cells may reside in the methylcellulose as single cells and not only within the colonies, we compared the procedure of harvesting the whole culture dish with a known number of colonies with that of pooling the same number of colonies collected one by one with a micropipette. In four separate experiments the mean recloning values were 5.56 ± 1.50 for the first method and 5.99 ± 2.32 for the second method of recloning. These results indicated that the secondary colonies mainly arise from cells residing within the colonies formed on day 7.

Frequency distribution of normal colony-forming cells

In three cases single colonies were transferred to separate culture wells for analysis of the frequency distribution of colony forming cells within colonies formed after seven days. Figure 2 shows the cumulative distribution of colony-forming cells in 89, 91 and 45 colonies, which gave rise to 283, 640 and 392 secondary colonies respectively (A, B and C). The distributions were heteroge-

neous and in only one case (C), could the experimental data be fitted to an expected exponential distribution; in the two other cases (A and B) there were too many primary colonies without any secondary colonies to allow a fit to an exponential distribution. In case A, 45/89 colonies contained zero secondary colonies and the corresponding figures were 36/91 and 6/45 in cases B and C respectively. Primary colonies with 30 or more secondary colony-forming cells were rare; 2 in case A and 4 in cases B and C.

Recloning of colony-forming cells in CML

In contrast to normal marrow cells, day 7 colonies derived from marrow or blood from CML patients were defective in their recloning capacity. The number of replated and secondary colonies are shown in table 1, and in fig. 1 the calculated recloning values are compared with those of normal marrows. The recloning capacity was almost identical for blood and marrow in those patients where both tissues were studied. Only one patient had a value within the normal range on one occasion. Five of the patients were studied shortly after diagnosis before any treatment was given and they all had severely decreased recloning capacity, 0, 1.28, 0.22, 0.43, and 0.07 respectively (patients 1-5, table 1). Two patients were studied twice with an interval of nine and five months respectively. One of the patients (no. 6) had been treated for three weeks with busulfan shortly after diagnosis but had been off treatment for six months at the time of the first study; the recloning capacity was normal on this occasion but nine months later, after continuous therapy with busulfan, the recloning capacity was reduced to subnormal levels. The other patient (no. 11) had subnormal values on both occasions. The difference between normal and CML cells in recloning ability of CFU-GM was highly significant ($p < 0.001$, Student's t-test. HP-CM and GCT-CM were equally effective to sustain colony growth and recloning capacity (Table 2). These results indicate that in a majority of cases of CML the recloning capacity of day 7 CFU-GM is absent or severely reduced compared with normal CFU-GM and that this defect is present already at the time of diagnosis and before any treatment has been given.

Velocity sedimentation

When cells obtained from velocity sedimentation in albumin gradients were cultured, colonies were counted on day 7 and day 14 for construction of the size distribution of CFU-GM day 7 and CFU-GM day 14 respectively. On day 7 a known number of colonies from each fraction were pooled and replated for analysis of the recloning capacity. Figures 3 and 4 show the results from separations of normal and CML marrow cells. In both cases a partial separation between CFU-GM day 7 and day 14 was obtained and the recloning ability was

expressed almost entirely by slowly sedimenting cells within the peaks of CFU-GM day 14. The two other cases behaved similarly with the colony-forming cells with recloning capacity confined to the peak of CFU-GM day 14 (data not shown).

DISCUSSION

Extensive self-renewal capacity is normally restricted to early hemopoietic stem cells (7-10) and granulocyte-macrophage colony forming cells are generally considered to have low self-replicating capacity (11). In this work we demonstrate that normal marrow derived CFU-GM cultured in methylcellulose express a restricted self-replication on transfer of single or pooled colonies to fresh culture medium. The source of colony-stimulating factor did not seem to be critical since both HP-CM and GCT-CM were equally effective. In most experiments we used a simplified method for replating of colonies by pooling the entire cell population from the culture dishes. However, this method proved to be valid since a comparison of this method with the method of pooling single colonies gave similar results. It is concluded that the majority of secondary colonies arise from cells within the colonies harvested on day 7 and only a minority from dispersed single cells or clusters.

The cumulative frequency distribution of CFU-GM among day 7 colonies did not, with one exception, fit exponential distribution curves as has been shown for CFU-S (17) and a stem cell population identified in in vitro cultures (10) in similar analyses. This was due to the relatively high frequencies of day 7 colonies that did not contain any colony-forming cells. However, the distribution of those colonies containing one or more colony-forming cells did follow an exponential distribution. These results suggest that the pooled day 7 colonies is a mixture of two populations, one population that has no self-replicating capacity and another population that has a variable but restricted recloning capacity. Separation of normal marrow cells in velocity sedimentation has indicated that normal CFU-GM is a heterogeneous population consisting of at least two separable subpopulations, one that shows maximum colony formation on day 7 and another that shows maximum colony formation on day 14 of culture (18). Detailed studies have indicated that CFU-GM day 14 may give rise to CFU-GM day 7 (18). This relationship suggested that there may be a difference in the recloning ability between CFU-GM day 7 and day 14, which we were able to demonstrate by recloning of marrow cells separated by velocity sedimentation. These experiments showed that it is mainly CFU-GM day 14 that has the capacity

to generate new colony-forming cells in this culture system. We are aware of the fact that this capacity is not an expression of self-renewal of stem cells in the strict sense of the word, and the experiments we performed to reclone secondary colonies showed that serial transfer of granulopoietic colonies grown in methylcellulose is not possible. On the other hand, it is noteworthy that a minority of the colonies harvested on day 7 contained 30-54 colony-forming cells.

With regard to patients with CML we found a defective recloning capacity of colonies derived from marrow or blood. In only one of 13 cases did we find a normal recloning capacity and when this patient was studied again nine months later the recloning capacity was reduced to subnormal levels. Both blood and marrow derived colonies had defective recloning and there was no obvious relation to either WBC or the duration of the disease. It has been shown that busulfan impairs the self-renewal capacity of murine primitive stem cells (19) and it was first suspected that the reduced recloning capacity in CML was an effect of busulfan therapy. However, we found subnormal recloning capacities in five patients with newly diagnosed CML before any treatment was given. It therefore seems that reduced recloning capacity among CFU-GM in CML is inherent to the disease.

Evidence has been presented that small slowly sedimenting granulocyte-macrophage progenitors in CML express an accelerated maturation and a limited proliferative capacity (20), which may be in line with our observations of a reduced recloning ability in CML. However, the data are difficult to compare because in this earlier study (20) all cell aggregates of more than two cells were counted as colonies, whereas we have used a lower limit of 40 cells to define a colony. It can be argued that the discrepancy observed between normals and CML patients may depend on an altered distribution of CFU-GM day 7 and day 14 in CML; for instance, if the majority of CFU-GM in CML are CFU-GM day 7 this could explain the very low frequency of colony-forming cells among day 7 colonies. However, this does not seem to be the case. A comparison of the number of colonies on day 7 and day 14, including data from a previous study (12), showed that the number of colonies increased in normals with a mean of 28 % from day 7 to day 14 and decreased in CML cultures with a mean of 15 %. This difference does not explain the extremely low recloning capacity in CML patients. Furthermore, the velocity sedimentation experiments showed that CFU-GM day 7 and day 14 are separable also in CML and in one case the number of colonies on day 14 by far exceeded the number of colonies on day 7 (fig. 4) accompanied by a severely reduced recloning capacity.

There is evidence to suggest that the stem cell compartment is structured based on the generation age of the cells (19, 21). This model implies that each stem cell has a limited number of self-replicative generations and that with each generation the probability for self-renewal decreases and the probability for terminal differentiation increases. It is not known if the probability for self-renewal is altered among early hemopoietic stem cells in CML. The intriguing finding in a recent report (22) that cells belonging to the Ph¹-chromosome positive clone disappeared during long-term marrow culture, whereas normal marrow cells survived for periods of two to three months may suggest a defective self-replicating capacity in CML stem cells. Hypothetically, a decreased probability for self-renewal in CML would lead to an increased probability for terminal differentiation and could thus contribute to the granulocytic hyperplasia, but would ultimately lead to an exhaustion of the stem cell pool. Whether the decreased recloning capacity that we have observed for committed granulopoietic stem cells in CML is a reflection of an altered probability for self-renewal present also in early stem cells in CML could possibly be elucidated by studies of the self-replicating ability of mixed colonies such as CFU-GEMM.

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Table 1.

Clinical data and recloning ability of CFU-GM in chronic myeloid leukemia.

Patient	Sex/Age	Duration of disease (mos)	Treatment ⁽¹⁾	WBC $\times 10^{-9}/l$	Recloning ability ⁽²⁾ marrow	Recloning ability ⁽²⁾ blood
1.	F/50	0.5	none	120	52	0
2.	F/55	0.5	none	220	986	1261
3.	M/63	0.5	none	355	809	180
4.	M/66	0.5	none	35	606	258
5.	M/69	1	none	95	641	43
6a.	F/60	7	none ⁽³⁾	128	-	388
6b.	"	16	B (2mg/d)	98	645	387
7.	F/78	24	B (6mg/w)	52	115	40
8.	F/61	33	H (5g/w)	15	1413	106
9.	M/30	40	B (18mg/w)	17	-	469
10.	F/34	41	none ⁽⁴⁾	25	138	29
11a.	F/69	66	B (4mg/w)	90	-	331
11b.	"	71	" "	82	6	0
12.	F/69	96	B (4mg/w)	35	410	32
13.	M/41	265	B (4mg/w)	39	11	0

(1), treatment at the time of study, patients 1-5 had got no treatment at all; B = busulphan, H = hydroxyurea; (2), the left column show the number of pooled colonies replated on day 7, and the right column show the number of secondary colonies for marrow and blood respectively; (3), treated with busulphan for three weeks six months before the day of study; (4), treatment with busulphan was interrupted three weeks before the study

Table 2

Recloning ability with two different sources of CSF

	HP-CM		GCT-CM	
1. normal b.m.	610	2836 (4.65)	562	2715 (4.83)
2. normal b.m.	526	1201 (2.28)	557	1621 (2.91)
3. CML b.m.	986	1261 (1.28)	793	1065 (1.34)
blood	605	727 (1.20)	515	800 (1.55)
4. CML b.m.	645	387 (0.60)	624	170 (0.27)
blood	784	654 (0.83)	767	189 (0.25)

HP-CM, human placenta conditioned medium; GCT-CM, conditioned medium from the human monocyte-like cell line GCT; both HP-CM and GCT-CM were tested at 10% final concentration; the left columns show the number of day 7 colonies replated and the right columns show the number of secondary colonies on day 7; the figures in brackets show recloning ability expressed as the number of secondary colonies produced per replated colony.

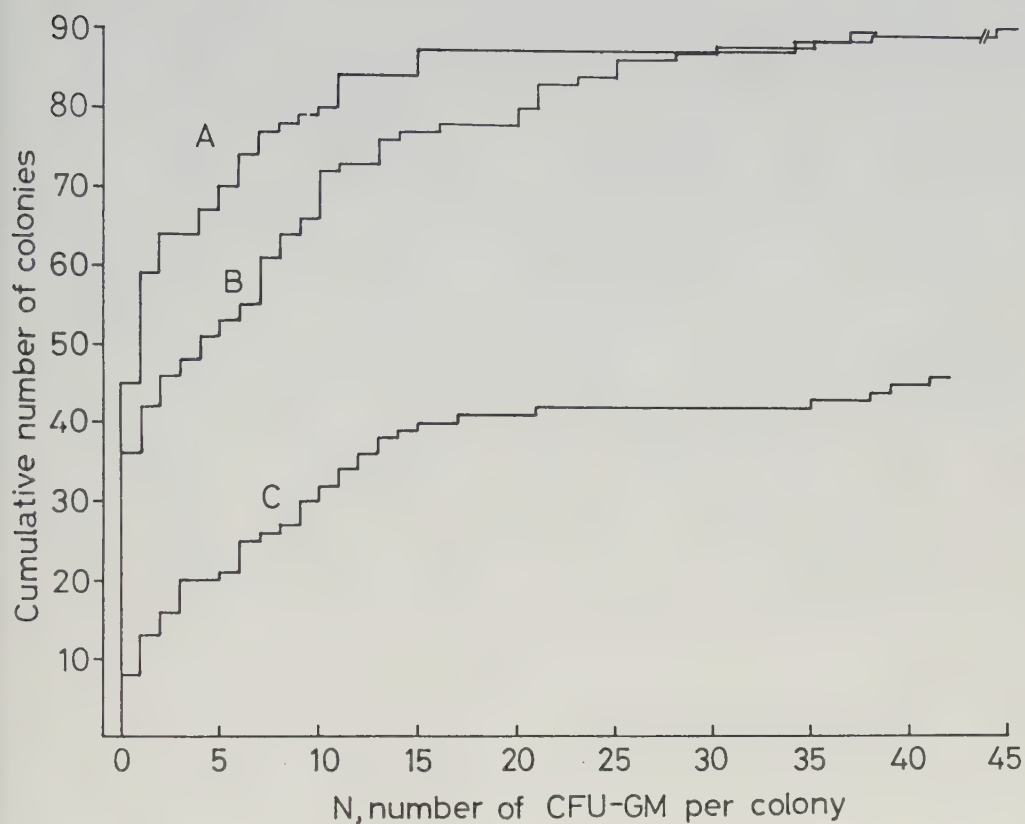


Fig. 2

Cumulative frequency distribution of CFU-GM in colonies grown for 7 days in methylcellulose. Three normal cases are shown. Curve A shows the data from 89 single colonies producing a total of 283 secondary colonies; the corresponding figures for curve B were 91 and 640, and for curve C 45 and 392 respectively.

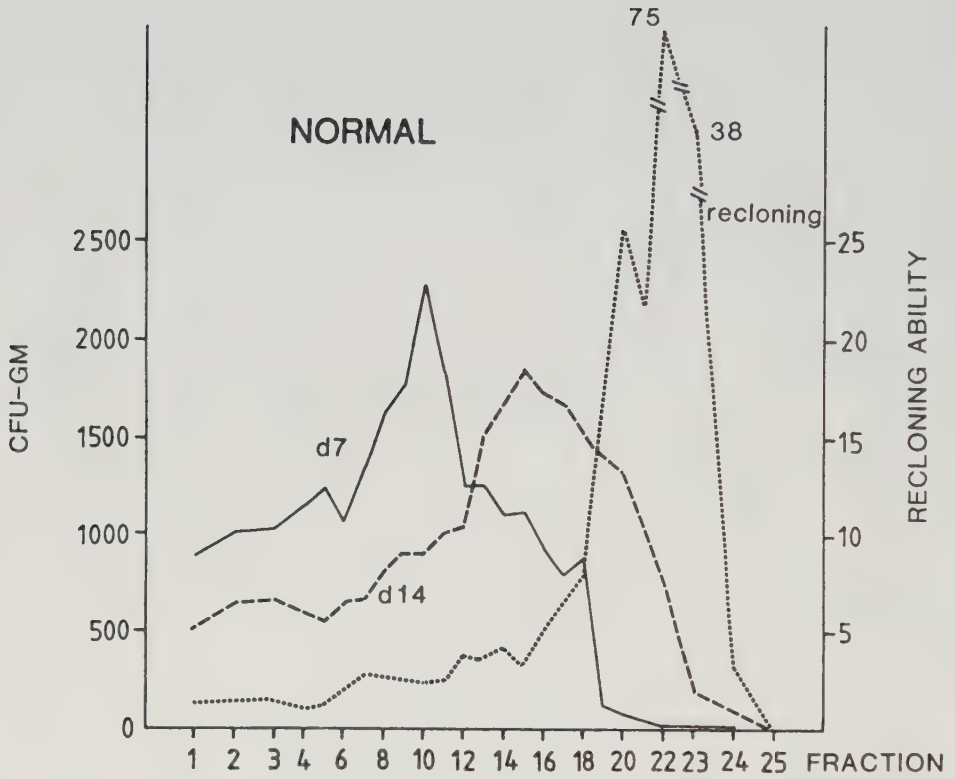


Fig. 3

Distribution of normal CFU-GM day 7 and CFU-GM day 14 and the recloning capacity of the colony forming cells in each cell fraction after separation of mononuclear cells with velocity sedimentation. Higher fraction number denotes lower sedimentation velocity. The recloning capacity was calculated as described in fig. 1.

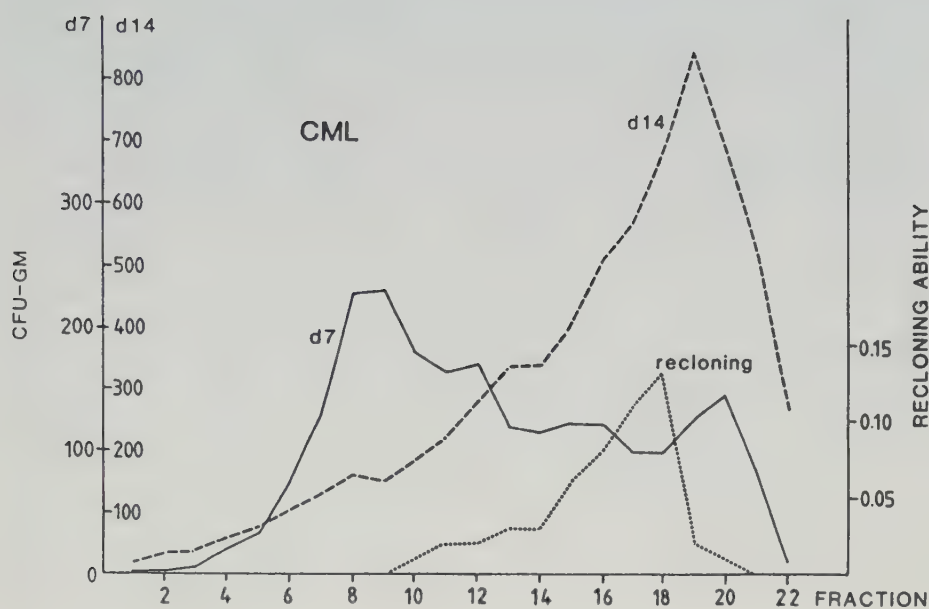


Fig. 4

Distribution of CFU-GM day 7 and CFU-GM day 14 from a patient with CML and the recloning capacity of the colony forming cells in each cell fraction after separation of mononuclear cells with velocity sedimentation. Higher fraction number denotes lower sedimentation velocity. The recloning capacity was calculated as described in fig. 1.

Probable *in vivo* induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukaemia

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SUMMARY. A 30-year-old woman was diagnosed as having an acute promyelocytic leukaemia in September 1981. Chemotherapeutic courses of daunomycin, ara-C, thioguanine and prednisolone were administered, resulting in a complete remission. A relapse occurred in January 1982, and chemotherapy did not lead to a second complete remission; neutropenia persisted with a marked left shift of the marrow granulopoiesis. Courses of chemotherapy were given throughout the study. In September 1982, marrow promyelocytes markedly increased, making up 51% of the nucleated cells. One month later the situation was unchanged, and 13-cis-retinoic acid (1 mg/kg) was administered by mouth. Gradually the marrow proportion of promyelocytes decreased to normal levels. The peripheral blood and marrow were still normal after 20 weeks of treatment with retinoic acid. Thus, retinoic acid seemed to have been inducing differentiation in an abnormally increased, maturation-deficient population of promyelocytes in a patient with acute promyelocytic leukaemia.

Acute promyelocytic leukaemia (APL) is a subgroup of acute myeloid leukaemia, usually characterized by bone marrow dominance of hypergranular promyelocytes, often containing multiple Auer rods (Bennet *et al.*, 1976). The disease is typically associated with disseminated intravascular coagulation (DIC), accompanied by haemorrhagic complications (Gralnick *et al.*, 1972; Sultan *et al.*, 1973). The marrow cells carry a unique chromosome abnormality in at least 50% of the patients, t(15q+;17q-) (Rowley *et al.*, 1977).

Retinoic acid (RA) is a metabolite of retinol, which is more rapidly metabolized and excreted than vitamin A (Brazell & Colburn, 1982). It has previously been used in the treatment of severe cystic acne without severe side effects (Peck *et al.*, 1979). Retinoids have been shown to play a role for the differentiation of epithelial cells (Clamon *et al.*, 1974; Hicks, 1975; Moore, 1976), and a chemoprotective effect in chemical carcinogenesis has been demonstrated in animal models (Sporn *et al.*, 1976). RA induces differentiation of the human promyelocytic cell line HL-60 to granulocytes (Breitman *et al.*, 1980), even in the presence of

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growth-inhibiting concentrations of hydroxyurea or ara-C (Ferrero *et al*, 1972). In primary culture, RA also induces differentiation of fresh cells from patients with APL, but not from patients with other types of acute leukaemia (Breitman *et al*, 1981).

In this paper, a patient with APL is reported, where maturation-blocked promyelocytes were induced to differentiate by the administration to the patient of RA.

CASE REPORT

The patient, a 30-year-old housewife, was well until August 1981, when she felt an increasing fatigue and eventually, easy bruising. Her haemoglobin was 6.3 g/dl, WBC 5500 per mm^3 , and the platelets 20 000 per mm^3 (Fig 1). The marrow smear was hypercellular and dominated by promyelocytes, most of which were hypergranular, containing multiple Auer rods (Fig 2). A karyotype analysis of the bone marrow cells disclosed the $t(15q+;17q-)$ abnormality. A diagnosis of APL was made, and therapy with daunomycin, 60 mg/m^2 i.v. day 1, ara-C, 70 mg/m^2 i.v. every 12 h days 2–6, thioguanin, 70 mg/m^2 orally every 12 h days 2–6, and prednisolone, 30 mg/m^2 orally days 2–6, was instituted. Shortly, disseminated intravascular coagulation occurred, manifested as massive haematuria, which was successfully treated with daily infusions of plasma, platelets and low-dose heparin, and erythrocytes as needed. In November 1981 the marrow was hypoplastic and the patient had severe

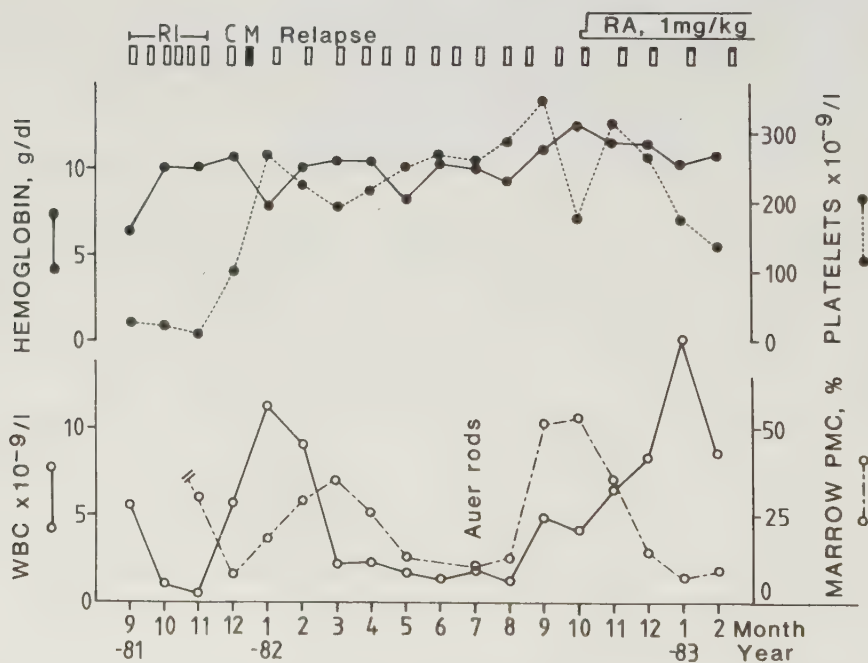


Fig 1. Changes in blood and marrow cell counts during the course of the disease. R = remission induction, C = consolidation, M = maintenance, denotes courses of chemotherapy (see text for details), RA = retinoic acid, PMC = promyelocytes.

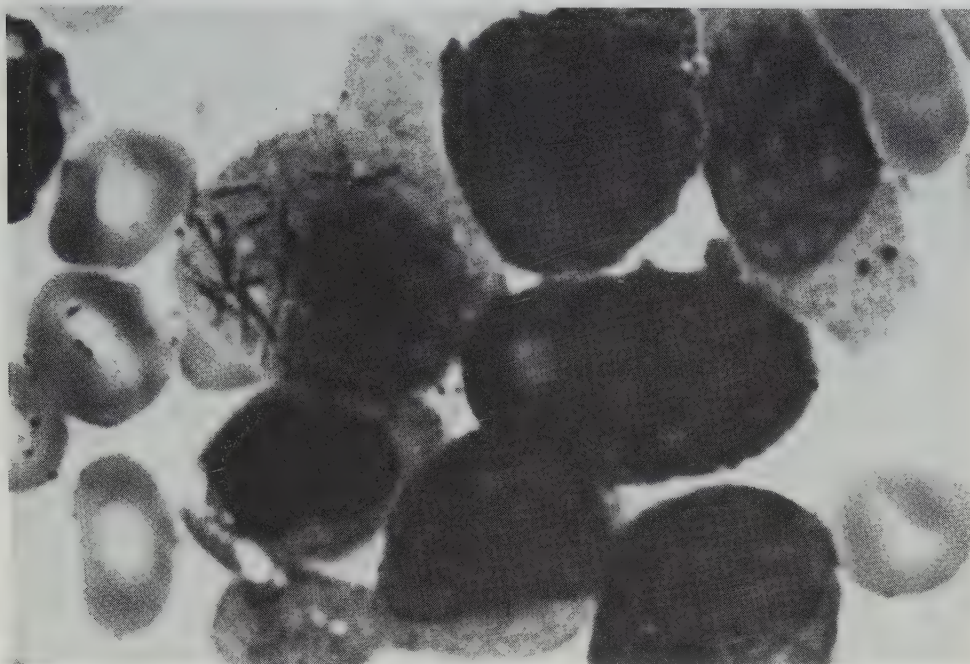


Fig 2. Marrow smear at diagnosis. $\times 75$. Promyelocytes, hypergranular or containing multiple Auer rods, were dominating.

pancytopenia. A few episodes of septic fever were successfully treated with antibiotics. Eventually, the marrow cells regenerated, and the patient went into complete remission. One course of consolidation (identical to remission induction courses) was given, and maintenance treatment with cyclophosphamide, 100 mg/m² orally days 1–5, vincristine, 2 mg i.v. day 1, ara-C, 100 mg/m² subcutaneously days 1–5, and prednisolone, 200 mg orally days 1–5, was started. However, in January 1982 signs of relapse occurred and chemotherapy identical to that during the first remission induction was given. A complete second remission was, however, not obtained. The haemoglobin and platelets were normal, but a neutropenia of about 1000 per mm³ persisted with a marked shift to the left of the granulopoiesis in the bone marrow (Fig 3). Intermittent courses with daunomycin, ara-C, thioguanin and prednisolone were given, but daunomycin was abandoned in September 1982 because of echocardiographic signs of myocardial damage, which was not clinically noticeable. At the same time a marked increase of the marrow promyelocytes was noted, comprising 51% of the nucleated cells in the marrow (Fig 4). The peripheral blood counts were normal. The situation remained unchanged for 1 month with 53% promyelocytes in the marrow. No Auer rods were seen. The chromosome analysis was normal. The chemotherapeutic regimen with 5 d courses of ara-C and thioguanin monthly continued throughout this study.

From October 1982, 13-cis-RA, 1 mg/kg/d, orally (40 mg twice daily) was administered. The peripheral blood counts remained normal, but the marrow proportion of promyelocytes

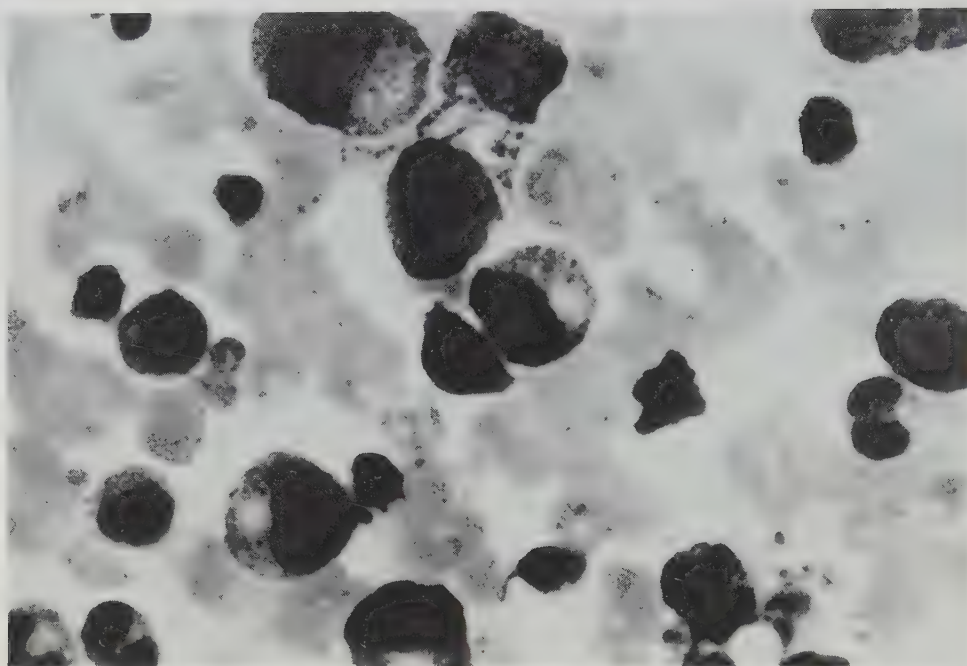


Fig 3. Marrow smear from May 1982. $\times 30$. Granulopoiesis is sparse with a marked left shift, erythropoiesis is dominating.

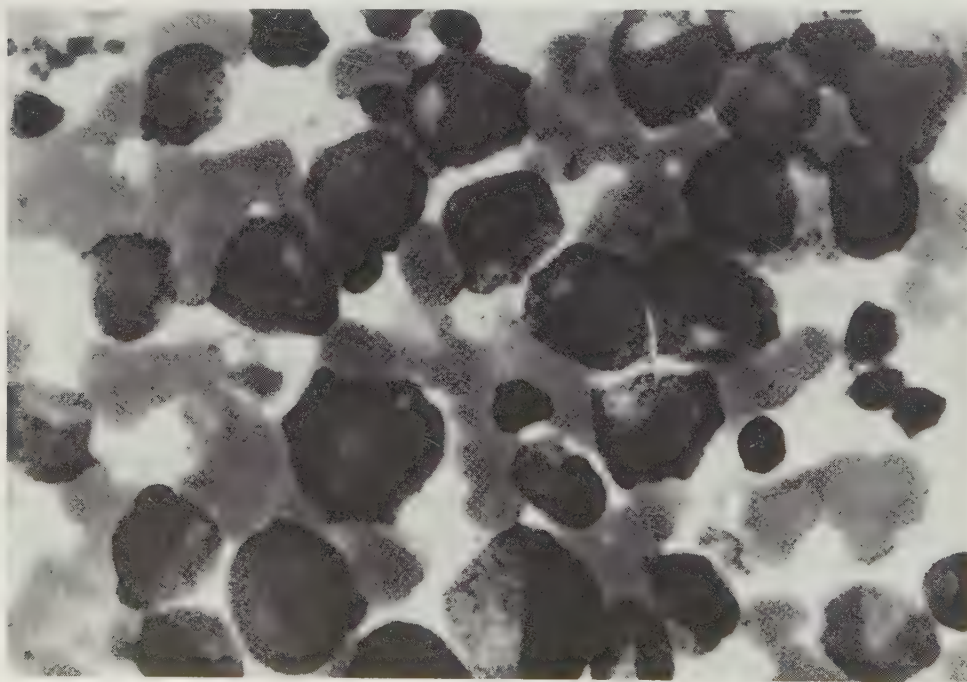


Fig 4. Marrow smear from October 1982. Highly cellular marrow, promyelocytes making up 53% of nucleated cells. Immediately before the institution of therapy with retinoic acid.

decreased gradually and remained at a normal level after 16 weeks of treatment with RA (Fig 1). At this time the marrow was slightly hypercellular with normal proportions of nucleated cells (Fig 5). Haemoglobin was 10.7 g/dl, WBC 8500 per mm³ and platelets 150 000 per mm³. The side effects of RA were a slight desquamation of the skin and conjunctivitis.

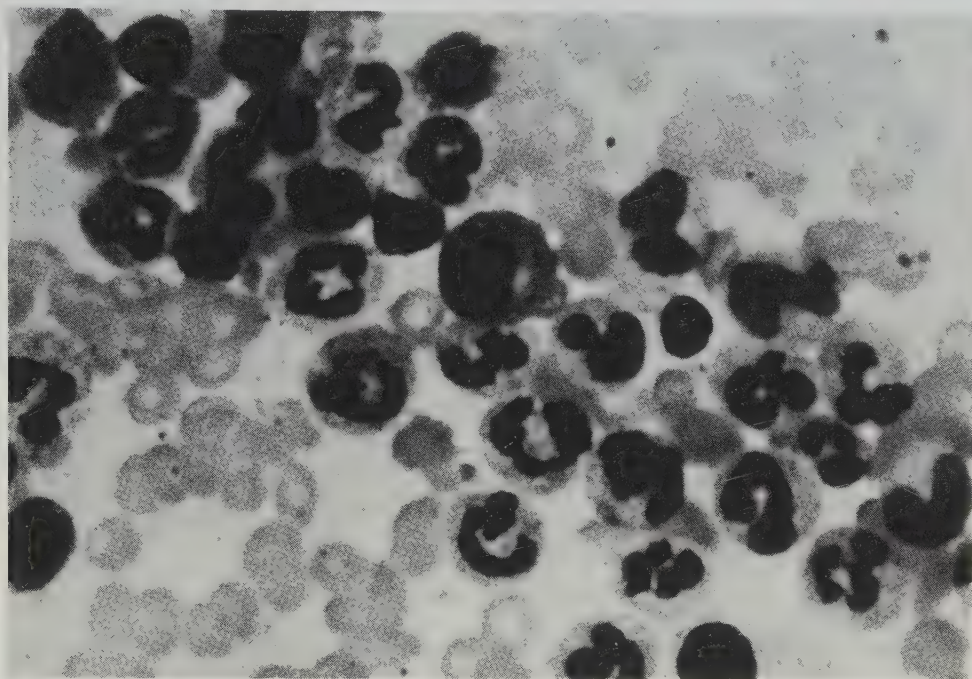


Fig 5. Marrow smear from January 1982. Highly cellular smear with normal composition of nucleated cells, compatible with a complete remission.

DISCUSSION

In this patient with APL in relapse, a population of maturation-deficient promyelocytes disappeared or matured to normal-appearing granulocytes during the administration of RA. Spontaneous differentiation seems unlikely since the maturation defect of promyelocytes had persisted for 10 months prior to the administration of RA. At diagnosis, the leukaemic cells carried the typical chromosomal abnormality, while the karyotype analysis in September 1982 was normal. Therefore it was not possible to decide if the accumulated promyelocytes were leukaemic or normal. Anyhow, they seemed to be blocked in differentiation. The normal karyotype may reflect coexisting normal cells, or the karyotype of the leukaemic cells normalized by the chemotherapy, or changed because of clonal evolution. It is possible although unlikely, that the disappearance of promyelocytes during RA administration was spontaneous and part of the natural course of the disease. In addition, an effect of the chemotherapy administered cannot be completely ruled out. The long duration of the maturation block of promyelocytes in spite of continuous pulse chemotherapy favours a role

of RA as responsible for the differentiation induction. The fact that the disappearance of immature cells was not clearly demonstrated until after several weeks of treatment with RA may have several explanations: RA was not responsible for the effect, the effect of RA was indirect, or the dose of RA was not optimal. However, in the light of the previously described effects of RA *in vitro* on differentiation induction of leukaemic promyelocytes (Breitman *et al.*, 1980, 1981), it is possible that a similar effect in this patient could be obtained *in vivo* and explain the effects reported.

The mechanisms responsible for induction of differentiation by RA are not understood. Cell division did not seem to be a requirement for differentiation induction of APL cells *in vitro* (Breitman *et al.*, 1981). The proliferation of normal marrow cells in culture seems, however, to be enhanced by RA, possibly by an increased sensitivity to colony stimulating activity (Douer & Koeffler, 1982). Altered membrane glycosylation by RA may be of importance for differentiation induction (Wolf *et al.*, 1979). The prognosis of the patient described has to be judged with great care because residual leukaemic cells that are resistant to both cystostatic and differentiation inducing therapy may occur, or because of possible clonal evolution. However, this case report at least suggests the possibility of inducing differentiation *in vivo* of haemopoietic precursor cells in some patients with leukaemia.

Addendum. In September 1983 the patient is still well with normal marrow and blood counts after a short period of transient moderate anaemia and thrombocytosis. The treatment has been unchanged.

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